Genetics of hemolytic uremic syndrome

GENETICS OF HEMOLYTIC UREMIC SYNDROME

Marina Noris

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PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus,
Prof.mr. G.P.M.F. Mols,
Volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op donderdag 16 Maart 2006 om 16.00 uur

door

Marina Noris
geboren op 20 July 1961 te Alzano L. (Bergamo), Italie

Supervisors

Prof. dr. K.L.M. Leunissen

Prof.dr. G. Remuzzi, Bergamo

Beoordelingscommissie

Prof.dr. H. ten Cate, (voorzitter)

Prof.dr.T. Goodship, Newcastle, UK

Prof.dr. J. Rosing

Prof.dr. C. Schrander-Stumpel

Prof.dr. P.F. Zipfel, University of Jena, Germany.

To: *Daniele, Andrea and Francesco*

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CHAPTER 1

Introduction

In part published in the review:

Disease of the month. Hemolytic uremic syndrome

Journal of the American Society of Nephrology 2005;16:1035-1050

Definition

Hemolytic uremic syndrome (HUS) is a rare disease of microangiopathic hemolytic anemia, low platelet count and renal impairment due to platelet thrombi occluding the renal circulation [1]. Anemia is severe, non-immune (Coombs negative) and microangiopathic in nature, with fragmented red blood cells (schistocytes) in the peripheral smear, high serum lactate dehydrogenase (LDH), circulating free hemoglobin and reticulocytes. Platelet count is lower than 60,000/mm^3 in most cases [1] and platelet survival time is reduced, reflecting enhanced platelet disruption in the circulation.

The common microvascular lesion of HUS, defined by the term thrombotic microangiopathy, consists of vessel wall thickening (mainly arterioles and capillaries) with endothelial swelling and detachment, which allows accumulation of proteins and cell debris in the subendothelial layer, creating a space between endothelial cells and the underlying basement membrane of affected microvessels [1, 2]. Both the widening of the subendothelial space and intraluminal platelet thrombi lead to a partial or complete obstruction of the vessel lumina. It is probably because of the partial occlusion of the lumen that erythrocytes are disrupted by mechanical trauma, which explains the Coombs-negative hemolysis and finding of fragmented and distorted erythrocytes in the blood smear. In children younger than 2 years of age the lesion is mainly confined to the glomerular tuft and is noted in an early phase of the disease. Examination of biopsies taken several months after the disease onset showed that most glomeruli were normal (an indication of the reversibility of the lesions), whereas 20% eventually became sclerotic [3, 4]. Arterial thrombosis do occur but is

uncommon and appears to be a proximal extension of the glomerular lesion [3, 4]. In adults and older children, glomerular changes are different and more heterogeneous than in infants. The classical pattern of thrombotic microangiopathy is less evident. In these patients glomeruli mainly show ischemic changes with retraction of glomerular tufts, expansion of the urinary space, and thickening and wrinkling of the capillary wall[5]. When the renal biopsy is performed in the early phase of the disease, changes may be reminiscent of those of younger children with typical thickening of the glomerular capillary wall and swelling of the endothelium. Thrombi, packed and fragmented red blood cells are observed in the capillary lumina. Occasionally, glomeruli have necrotic areas or segmental extracapillary proliferation. Late in the course of the disease, thickening of the glomerular basement membrane and intraglomerular thrombi are less frequent and the ischemic changes prevail [4].

In children with a predominantly glomerular pattern, arteriolar lesions are rather heterogenous: mild arteriolar lesions with some vessels even not affected by the microangiopathy were reported in a study [6], but arteriolar thrombosis was frequently detected in other childhood series [7, 8] with similar glomerular pattern. In adults, changes in the arterioles and arteries are common with severe narrowing of the lumina due to expansion of the subendothelial space. Fibrin and platelet thrombi and myointimal proliferation also are frequent. Vascular lumina often are congested with sludged and fragmented red blood cells. [3, 4]. Red blood cells and fibrin also may infiltrate the walls of arteries. Intimal proliferation has been regarded as a response to intimal swelling and to the accumulation of red blood cells and fibrin. When specimens are examined in the late phase

of the disease, intimal thickening is replaced by dense intimal fibroplasias which leads to marked narrowing of the lumina.

Stx-associated HUS

In children the disease is most commonly triggered by certain strains of *E. coli* that produce powerful exotoxins, the Shiga-like toxins (Stx1 and Stx2) [9-13]. In 70% of cases in North America and Western Europe Stx-HUS is secondary to infection with the *E.coli* serotype O157:H7 [9-11, 14-17]. However, many other *E.coli* serotypes (O111:H8, O103:H2, O121, O145, O26, and O113 [9, 14, 18-20]), have been shown to cause Stx-HUS. The disease manifests with diarrhea (D^+HUS), often bloody. Cases of Stx-*E.coli* HUS -around 25% [21]- which however do not present with diarrhea, have also been reported [2]. Acute renal failure manifests in 55%-70% of cases [22-24], however renal function recovers in most of them (up to 70% in various series) [1, 2, 24, 25].

Following exposure to Stx-*E.coli*, 38 to 61% of individuals develop hemorrhagic colitis and 3-9% (in sporadic infections) to 20% (in epidemic forms) progress to overt HUS [12, 23].

Humans become infected by Stx-producing *E.coli* from contaminated milk, meat and water -water-borne outbreaks have occurred as a result of drinking and swimming in unchlorinated water [18]- or from contact with infected animals, humans or either's excreta [12, 26, 27] and occasionally through environmental contamination [15].

The disease is characterized by prodromal diarrhea followed by acute renal failure The average interval between *E.coli* exposure and illness is three days (range of 1 to 8). Illness

typically begins with abdominal cramps and non bloody diarrhea; diarrhea may become hemorrhagic in 70% of cases usually within one or two days [28]. Vomiting occurs in 30 to 60% of cases and fever in 30%. HUS is usually diagnosed six days after the onset of diarrhea [1]. After infection, Stx-*E.coli* may be shed in the stools for several weeks after the symptoms are resolved, particularly in children <5 years of age [1]. Diagnosis rests on detection of Stx-*E.coli* in stool cultures. Serologic tests for antibodies to Stx and O157 lipopolysaccharide can be done in research laboratories and tests are being developed for rapid detection of *E.coli* O157:H7 and Stx in stools.

Bloody diarrhea, fever, vomiting, elevated leukocyte count, extremes of age and female sex as well as the use of antimotility agents [29], have been associated with an increased risk of HUS following *E.coli* infection [12] .

Stx-HUS is not a benign disease. Seventy-percent of patients who develop HUS require red blood cell transfusions, 50% need dialysis, and 25% have neurological involvement, including stroke, seizure and coma [12, 24, 30]. Although mortality for infants and young children in industrialized countries decreased when dialysis became available, as well as after the introduction of intensive care facilities, still 3% to 5% of patients die during the acute phase of Stx-HUS [24]. A recent meta-analysis of 49 published studies (3476 patients, mean follow-up of 4.4 years) describing long-term prognosis of patients who survived an episode of Stx-HUS, reported death or permanent end-stage renal disease (ESRD) in 12% of patients and GFR below 80 ml/min/1.73m^2 in 25% [30]. The severity of acute illness, particularly central nervous system symptoms, and the need for initial dialysis were

strongly associated with a worse long-term prognosis [22, 30].

Non-Shiga toxin HUS

Non-Shiga toxin-associated HUS (non-Stx-HUS) comprises a heterogeneous group of patients in whom an infection by Stx-producing bacteria could be excluded as cause of the disease. It can be sporadic or familial (i.e. more than one member of a family affected by the disease and exposure to Stx-*E.coli* excluded). Collectively, non-Stx-HUS forms have a poor outcome. Up to 50% of cases progress to ESRD or have irreversible brain damage, and 25% may die during the acute phase of the disease [31-33].

Extrarenal manifestation, including neurologic complications, are not uncommon so that non-Stx-HUS is often clinically undistiguishable from thrombotic thrombocytopenic purpura (TTP), a related thrombotic microangiopathy characterized by microvascular occlusive thrombosis in the brain and other organs.

Non-Stx-HUS is less common than Stx-HUS, and accounts for only 5 to 10% of all cases of the disease [1, 34]. It may manifest at all ages, but is more frequent in adults. According to a recent US study the incidence of non-Stx-HUS in children is approximately one tenth that of Stx-HUS [33], corresponding to approximately 2 cases/year/1,000,000 total population. At variance with Stx-HUS there is no clear etiologic agent or seasonal pattern. The onset may be preceded by features of the nephrotic syndrome. A diarrhea prodrome is rarely observed (D-HUS) [1, 2, 33, 35]. Non-Stx-HUS can occur sporadically or in families.

Sporadic non-Stx-HUS

A wide variety of triggers for sporadic non-Stx-HUS have been identified including various non-enteric infections, viruses, drugs, malignancies, transplantation, pregnancy and other underlying medical conditions (scleroderma, anti-phospholipid syndrome, lupus, malignant hypertension).

Infection caused by *Streptococcus pneumoniae* accounts for 40% of non-Stx-HUS and 4.7% of all causes of HUS in children in US [33]. Neuroaminidase produced by *Streptococcus pneumoniae*, by removing sialic acids from the cell membranes, exposes Thomsen-Friedenreich antigen to preformed circulating IgM antibodies, which bind to this neoantigen on platelet and endothelial cells and cause platelet aggregation and endothelial damage [36, 37]. The clinical picture is usually severe, with respiratory distress, neurological involvement and coma. The mortality rate is 50% [37].

HUS and the related syndrome, thrombotic thrombocytopenic purpura (TTP) are both part of the complications of AIDS following HIV infection and account for up to 30% of hospitalized cases of HUS and TTP in some institutions [38]. The clinical course is poor and depends heavily on the severity of the underlying disease.

Categories of drugs that have been most frequently reported to induce non-Stx-HUS include anti-cancer molecules (mitomycin, cisplatin, bleomycin, gemcitabine), immunotherapeutic (cyclosporine, tacrolimus, OKT3, interferon and quinidine), and antiplatelet (ticlopidine, clopidogrel) agents [39]. The risk of developing HUS after mitomycin is 2% to 10%. The onset is delayed, occurring almost 1 year after starting

treatment. The prognosis is poor, with up to 75% mortality at 4 months [39].

Post-transplant HUS is being reported with increasing frequency [1, 40]. It may ensue for the first time in patients who never suffered from the disease (de novo post-transplant HUS) or may affect patients whose primary cause of ESRD was HUS (recurrent post-transplant HUS). De novo post-transplant HUS might occur in patients receiving renal transplants and other organs, as a consequence of the use of calcineurin inhibitors or due to humoral (C4b positive) rejection. In renal transplant patients treated with cyclosporine the incidence of the disease is 5 to 15%; a lower incidence, approximately 1%, is found in patients receiving tacrolimus [41]. A peculiar form of de novo post-transplant HUS affects about 6% of the recipients of a bone marrow transplant (BMT), usually in the setting of a graft versus host disease (GVHD) or of intensive GVHD prophylaxis [41].

Non-Stx-HUS may also be triggered by pregnancy. Pregnancy-associated HUS may occasionally develop as a complication of pre-eclampsia. Some patients progress to a life-threatening variant of pre-eclampsia with severe thrombocytopenia, microangiopathic hemolytic anemia, renal failure and liver involvement (HELLP syndrome). HUS or its complications in pregnancy are always an indication for prompt delivery that is usually followed by complete remission [42]. Post-partum HUS is another complication of pregnancy that manifests within three months of delivery in most cases. The outcome is usually poor with 50-60% mortality; residual renal dysfunction and hypertension are the rule in surviving patients [43].

Of note, in about 50% of cases of sporadic non-Stx-HUS no clear triggering conditions

could be found (idiopathic HUS) [1]. The outcome is variable, it may follow a progressive course to ESRD, however many patients recover completely [1].

Familial non-Stx-HUS

Familial forms accounts for fewer than 3% of all cases of HUS. Reports of familial occurrence of HUS in children date back to 1965, when Campbell and Carre [44] described the development of hemolytic anemia and azotemia in concordant monozygous twins. Since then, familial forms of HUS in children and, less frequently, in adults, have been reported [45]. Although some reports have been in siblings, suggesting autosomal recessive transmission [46], there have also been others describing affected members across two [47-49] or three [46] successive generations, suggesting an autosomal dominant mode of inheritance. In a kindred [50], late onset in the grandmother and early onset in the grandson may be indicative of the phenomenon known as anticipation, in which clinical manifestations appear at an earlier age and with increasing severity in succeeding generations.

In autosomal recessive HUS the onset is usually early in childhood. The prognosis is poor, with a mortality rate of 60-70%. Recurrences are very frequent.

The onset of symptoms in autosomal dominant HUS varies –early in childhood in some cases, but it can manifests in adult life- and is often triggered by precipitating events such as bacterial and viral infections or pregnancy [46]. The use of oral contraceptives and the intake of estrogens have also been implicated as triggering factors [46]. Malignant hypertension is a frequent complication and the prognosis is poor, with a cumulative incidence of death or

ESRD of 50% [51, 52] to 90% [46].

Evidence that some familial cases of HUS responded, at least transiently, to plasma infusion or exchange led to the hypothesis that the genetic defect(s) associated with the disease might result in abnormalities in some plasma component(s) involved in the pathogenesis of the microangiopathic process. Biochemical and genetic studies from our group (included in this present thesis) and from other investigators have now provided evidence that familial HUS may be caused by genetic abnormalities of proteins involved in the regulation of the alternative pathway of complement .

The human complement system

The complement system is a crucial component of the innate immunity against microbial infection. It consists of several plasma and membrane-associated proteins that are organized in three distinct activation patterns. These include classical, lectin and alternative pathways [53, 54] that, once activated on the surface of microorganisms, form protease complexes, collectively known as C3-convertases, which serve to cleave C3 generating C3b. The classic/lectin C3 convertases, named C4b2b, are formed by C2 and C4 fragments, while the generation of the alternative pathway C3 convertase, named C3bBb, requires the cleavage of C3 and factor B but not of C4. Upon generation C3b deposits on bacterial surfaces, which leads to opsonization for phagocytosis by neutrophils and macrophages. In the presence of an additional C3b molecule, the C3-convertases form the C5 convertases that cleave C5 and initiate assembly of the membrane attack complex (MAC) that causes cell lysis.

The human complement system is highly regulated as to prevent non-specific damage to host cells and limit deposition of C3b to the surface of pathogens. This fine regulation is based on a number of membrane-anchored (CR1, DAF, MCP and CD59) and fluid-phase (factor H, factor I) regulators that protect host tissues. Foreign surfaces that either lack membrane-bound regulators or cannot bind soluble regulators are attacked by complement.

Factor H

Factor H (CFH) is encoded by a single gene (*CFH*) located on human chromosome 1q32 within the regulators of complement activation (RCA) cluster. It is a single polypeptide chain plasma glycoprotein (155 kDa) that is composed of 20 repetitive units of 60 aminoacids, named short consensus repeats (SCR) [55].

CFH binds to C3b, accelerates the decay of the alternative pathway C3-convertase and acts as a cofactor for the factor I-mediated proteolytic inactivation of C3b. In the presence of CFH and factor I, C3b proteolysis results in the cleavage of the alpha'-chain of C3b at two nearby sites generating two fragments of 68 and 43 kDa, respectively. In the case of surface-bound C3b, the inactivated C3b molecule, named iC3b, remains covalently linked to the activating surface. CFH regulates complement both in fluid-phase and on cellular surfaces. It binds and inactivates promptly C3b in fluid-phase, and complement regulatory domains that are needed to prevent fluid-phase alternative pathway activation have been localized within the N-terminal SCR1-4 [56]. On the other hand the inactivation of surface-bound C3b by CFH is dependent on the chemical composition of the surface to

which C3b is bound. Indeed, CFH binding to sialic acids and other polyanionic molecules like glycosaminoglycans or sulphated polysaccharides such as heparin, increases its affinity for surface-bound C3b and exposes its complement regulatory N-terminal domain. Human vascular endothelial cells and basement membranes are rich in polyanionic molecules, so that CFH bound on their surface would provide an efficient shield against complement attack [56].

CFH has three C3b-binding sites, located in SCR1-4, SCR12-14 and SCR19-20, respectively. Similarly, a total of three separate binding sites for heparin and sialic acid have been identified in SCR7, SCR13 and SCR19-20 [57]. The C3b binding site in SCR1-4 is the only site essential for the factor I cofactor activity of CFH in fluid-phase. On the other hand, the C3b and the polyanion-binding sites located in SCR19-20 are the only indispensable sites for CFH to inactivate surface-bound C3b, because deletion of this portion of the molecule causes loss of CFH capability to prevent complement activation on sheep erythrocytes (taken as an example of a host-like non-activator surface) [58].

Membrane cofactor protein

As *CFH*, the gene encoding membrane cofactor protein *(MCP)* is located within the RCA cluster on chromosome 1 with 14 exons spanning around 43 Kb [59].

MCP is widely expressed on the surface of almost every human cells except erythrocytes [59]. Structurally, MCP consists of four alternatively spliced isoforms that co-exists on most cells. The extra-cellular domain is composed of four SCR. Following this is

an O-glycosylated region that is rich in serines, threonines and prolines (the STP region), which is alternatively spliced. There are either 14 or 29 amino acids in the STP region and this depends on the splicing of STP exon B (STP-B). The STP region is followed by a juxtamembranous region of 12 amino acids of unknown function, followed by a hydrophobic transmembrane domain and a cytoplasmic anchor. The cytoplasmic tail of MCP is also alternatively spliced (the 16 amino acid CYT-1 and the 23 amino acid CYT-2) and mediates signalling events. There are four commonly expressed isoforms, MCP-BC1, MCP-BC2, MCP-C1 and MCP-C2 where B and C refer to the STP region, and 1 or 2 refer to the cytoplasmic tail.

MCP with factor I degrades the C3b and C4b that are bound to the cell surface (cofactor activity) and regulates both the classical and alternative pathways of complement. Studies with deletion mutants and monoclonal antibodies have shown that ligand binding and cofactor activity are contained in SCRs 2, 3 and 4 of MCP [59].

MCP is highly expressed in the kidney and could be found on glomerular endothelial cells by immunohistochemical analysis [60-62]. It likely exerts a main role in protecting glomerular endothelial cells against C3 activation as indicated by data that cofactor activity in the extracts of these cells was completely blocked by anti-MCP antibody [61].

Complement abnormalities in HUS, previous studies

Since 1974 reduced serum levels of the third component (C3) of complement have been reported in patients with both Stx-HUS and non-Stx-HUS, during the acute phase of the

disease [48, 63]. In patients suffering from episodes of Stx-HUS C3 abnormalities consistently subsided with remission of the disease. On the contrary, in cases of familial non-Stx-HUS and in patients suffering from recurrences, serum C3 levels were low even during remission of the disease [64, 65]. By contrast, levels of the fourth fraction of complement, C4, are usually normal. Low C3 levels most likely reflect complement activation and C3 consumption, which is consistent with increased levels of C3 breakdown products, C3b, C3c and C3d, in the plasma of these patients [66, 67]. Granular C3 deposits in glomeruli and arterioles of kidney biopsies taken during the acute phase of the disease [68-72] further reflect activation of the complement system and C3 consumption in the renal microvasculature.

In a patient and in his healthy brother as well, low C3 levels were accompanied by very low levels of CFH [73]. Finding that the parents, who were first cousins, had half-normal levels of CFH, convincingly indicated that the defect was inherited. Similar findings were reported in other families [65]. These reports raised the possibility that low C3 in familial HUS may depend on an inherited deficiency of CFH. However, epidemiological data on the prevalence of low C3 levels and CFH abnormalities in non-Stx-HUS were missing.

Objective of the thesis

Based on these observations a number of studies were designed and performed in this thesis with the aims to provide more information as for the incidence and pathogenetic role of complement disregulation in non-Stx-HUS and to investigate the genetic basis of such

an O-glycosylated region that is rich in serines, threonines and prolines (the STP region), which is alternatively spliced. There are either 14 or 29 amino acids in the STP region and this depends on the splicing of STP exon B (STP-B). The STP region is followed by a juxtamembranous region of 12 amino acids of unknown function, followed by a hydrophobic transmembrane domain and a cytoplasmic anchor. The cytoplasmic tail of MCP is also alternatively spliced (the 16 amino acid CYT-1 and the 23 amino acid CYT-2) and mediates signalling events. There are four commonly expressed isoforms, MCP-BC1, MCP-BC2, MCP-C1 and MCP-C2 where B and C refer to the STP region, and 1 or 2 refer to the cytoplasmic tail.

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abnormalities.

Sporadic observations in literature provided evidence that activation of complement occurs also in patients with thrombotic thrombocytopenic purpura (TTP). The latter syndrome has in common with HUS microangiopathic hemolytic anemia, consumption thrombocytopenia and microvascular thrombosis, but differs because it manifests mainly with central nervous system symptoms, whereas predominant renal involvement characterizes HUS [4]. Examination of very large series of patients with HUS and TTP has pointed out that a neat clinical distinction between the two syndromes is difficult. Thus, search for complement activation markers and of genetic abnormalities of complement regulatory genes was also undertaken in patients with diagnosis of TTP.

In chapter 2, in a case-control study we correlated putative predisposing conditions, including low C3 serum levels, with history of disease in familial cases of HUS and TTP. In the same subjects we searched for biochemical abnormalities in the complement regulatory plasma factor, CFH. The relationship between history of disease, low serum C3 and CFH abnormalities was also investigated.

Results of **chapter 2**, showed an association between CFH biochemical abnormalities and low C3 levels in familial forms of HUS. Based on these data, linkage and mutation studies were undertaken to evaluate whether mutations in *CFH* gene were involved in determining genetic predisposition to familial forms of HUS. Characterization of biochemical consequences of the mutations was also performed. For these studies, reported in **chapter 3**, four families were selected on the basis of persistent hypocomplementemia in

all cases.

Taking advantage of the large number of patients referred to the International Registry of HUS and TTP, a subsequent study (**chapter 4**) was designed to evaluate the frequency of *CFH* mutations both in familial cases of non-Stx-HUS and in sporadic cases as well, and to compare the clinical phenotype of patients with and without *CFH* mutations. Various polymorphisms in the promoter and in the coding regions of *CFH* gene have been described which may be associated with reduced CFH levels or activity. Thus a second aim of the study was to evaluate the frequency of *CFH* polymorphisms in patients with non-Stx-HUS as compared with a control population, to establish whether any of them segregated with the disease. The above analysis were also performed in patients with TTP to establish whether *CFH* gene mutations and polymorphisms may have a role in predisposing to TTP as well, or whether such abnormalities were specific for the HUS phenotype. **This is described in chapter 4.**

Recent studies provided evidence that 70 to 80% of cases of TTP are triggered by a deficiency of ADAMTS13, a plasma metalloprotease that cleaves von Willebrand factor multimers soon after their secretion by endothelial cells [74]. ADAMTS13 deficiency may be constitutive, as a result of homozygous or double heterozygous mutations in the corresponding gene or acquired, due to the presence of circulating inhibitory antibodies [74]. In two large studies[75, 76], deficiency of ADAMTS13 activity was found in patients with diagnosis of TTP but not in those with HUS. This observation generated the idea that a severe ADAMTS13 deficiency is enough to distinguish TTP from HUS. However, further

investigations based on large series of patients with HUS and TTP [77, 78] showed that even if most cases of thrombotic microangiopathy diagnosed as HUS due to the prevalence of renal symptoms have normal ADAMTS13, there are unequivocal cases of HUS characterized by low or undetectable ADMATS13 levels. In studies of **chapter 5** the genetic basis of phenotypic heterogeneity in patients with hereditary ADAMTS13 deficiency was investigated in a family with two sisters with complete ADAMTS13 deficiency, one with exclusive neurological symptoms that led to a diagnosis of TTP and the other with severe renal involvement more reminiscent of HUS.

Studies of **chapter 4** documented that two thirds of patients with non-Stx-HUS have no *CFH* mutations, despite up to 50% of them exhibit evidence of overactivity of the alternative pathway of complement. These findings indicated that genetic abnormalities in other complement regulatory proteins might be involved in determining genetic predisposition to the disease. Based on these observations, in studies of **chapter 6** patients with familial and sporadic forms of non-Stx-HUS with no *CFH* mutations were screened for mutations in genes encoding factor H related 5 (*FHR5*), complement receptor 1 (*CR1*) and membrane cofactor protein (*MCP*).

Finally, **chapter 7,** is a review chapter aimed at making the focus on the more recent acquaintances on the pathogenesis, the genetics and treatment of the different forms of HUS. The chapter also summarizes the results of expression studies we undertaken during the last years, in collaborations with Peter Zipfel in Jena, which were aimed at evaluating the functional consequences of the *CFH* mutations found in non-Stx-HUS on the activity of CFH

protein [79]. The outcome of combined kidney and liver transplant performed in our Transplant Center in two young children with non-Stx-HUS and *CFH* mutations with the objective of correcting the genetic defect and preventing disease recurrences [80, 81], is also reported and discussed .

CHAPTER 2

Hypocomplementemia discloses genetic predisposition to hemolytic uremic syndrome and thrombotic thrombocytopenic purpura: role of factor H abnormalities.

M. Noris, P. Ruggenenti, A. Perna, S. Orisio, J. Caprioli , C. Skerka,

B. Vasile, P.F. Zipfel and G. Remuzzi, published in

Journal of the American Society of Nephrology 1999;10:281-293

J Am Soc Nephrol 10: 281–293, 1999

Hypocomplementemia Discloses Genetic Predisposition to Hemolytic Uremic Syndrome and Thrombotic Thrombocytopenic Purpura: Role of Factor H Abnormalities

MARINA NORIS,* PIERO RUGGENENTI,*† ANNALISA PERNA,* SILVIA ORISIO,* JESSICA CAPRIOLI,* CHRISTINE SKERKA,‡ BEATRICE VASILE,* PETER F. ZIPFEL,‡ and GIUSEPPE REMUZZI,*† ON BEHALF OF THE ITALIAN REGISTRY OF FAMILIAL AND RECURRENT HEMOLYTIC UREMIC SYNDROME/THROMBOTIC THROMBOCYTOPENIC PURPURA[a]

**Mario Negri Institute for Pharmacological Research, Clinical Research Center for Rare Diseases "Aldo e Cele Daccò," Villa Camozzi, Ranica, Italy; †Unit of Nephrology and Dialysis, Azienda Ospedaliera, Ospedali Riuniti di Bergamo, Italy; and ‡Bernhard Noch Institute for Tropical Medicine, Hamburg, Germany.*

Abstract. Familial hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) carry a very poor outcome and have been reported in association with decreased serum levels of the third complement component (C3). Uncontrolled consumption in the microcirculation, possibly related to genetically determined deficiency in factor H—a modulator of the alternative pathway of complement activation—may account for decreased C3 serum levels even during disease remission and may predispose to intravascular thrombosis. In a case-control study by multivariate analysis, we correlated putative predisposing conditions, including low C3 serum levels, with history of disease in 15 cases reporting one or more episodes of familial HUS and TTP, in 25 age- and gender-matched healthy controls and in 63 case-relatives and 56 control-relatives, respectively. The relationship between history of disease, low C3, and factor H abnormalities was investigated in all affected families and in 17 controls. Seventy-three percent of cases compared with 16% of controls ($P < 0.001$), and 24% of case-relatives compared with 5% of control-relatives ($P = 0.005$) had decreased C3 serum levels. At multivariate analysis, C3 serum level was the only parameter associated with the disease within affected families ($P = 0.02$) and in the overall study population ($P = 0.01$). Thus, subjects with decreased C3 serum levels had a relative risk of HUS or TTP of 16.56 (95% confidence interval [CI], 1.66 to 162.39) within families and of 27.77 (95% CI, 2.44 to 314.19) in the overall population, compared to subjects with normal serum levels. Factor H abnormalities were found in four of the cases, compared with three of the healthy family members ($P = 0.02$) and none of the controls ($P = 0.04$) and, within families, factor H abnormalities were correlated with C3 reduction ($P < 0.05$). Reduced C3 clusters in familial HUS and TTP is likely related to a genetically determined deficiency in factor H and may predispose to the disease. Its demonstration may help identify subjects at risk in affected families.

Hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) are syndromes of microangiopathic hemolytic anemia and thrombocytopenia, which have in common thrombotic occlusion of the microvasculature of various organs (1). The term HUS is usually preferred to describe the disease in children with renal insufficiency (2), whereas TTP is the most used term to describe adult cases with predominant neurologic symptoms (3). However, it is now recognized that the two syndromes may have different clinical manifestations because of the different distribution of the microvascular lesions, but share the same histologic lesion—widening of the subendothelial space and intravascular platelet thrombi—and reflect a similar pathophysiologic process, leading to thrombocytopenia and anemia through platelet consumption and erythrocyte disruption in the injured microvasculature (4). In their typical presentation, HUS and TTP manifest as an acute disease that recovers without sequelae in 80 to 90% of cases, either spontaneously (as in most cases of childhood HUS) or following a course of plasma infusion or exchange (as in adult or severe forms of HUS and in TTP) (1–3). These forms may be triggered by environmental factors (as the verotoxins produced by some strains of *Escherichia coli*), drugs, or other diseases and may subside when the underlying condition has been treated or removed. Other forms, however, fail to recover or may relapse after complete recovery of the presenting epi-

Received April 20, 1998. Accepted September 4, 1998.

[a] See Appendix for participating investigators and affiliated organizations.

Dr. Richard J. Johnson served as Guest Editor and supervised the review and final disposition of this manuscript.

Correspondence to Dr. Piero Ruggenenti, Clinical Research Center for Rare Diseases "Aldo e Cele Daccò," "Mario Negri" Institute for Pharmacological Research, Via Gavazzeni, 11, 24125 Bergamo, Italy. Phone: 390 35 319888; Fax: 390 35 319331; E-mail: noris@irfmn.mnegri.it

1046-6673/1002-0281$03.00/0
Journal of the American Society of Nephrology

sode (1,5), with death or permanent neurologic or renal sequelae being the final outcome in the large majority of cases. In these "atypical" forms, an exogenous triggering factor is seldom recognized, and an underlying, genetically determined condition predisposing to the disease is hypothesized in most cases (6,7). Along these lines, an increased prevalence of HLA B40 antigens in a series of HUS cases has been taken to suggest that the HLA B40 genotype may be linked to "susceptibility genes" predisposing to the disease, possibly after exposure to exogenous precipitating factors (8).

Over the past 20 yr, about 140 cases of familial HUS and TTP have been described in 70 families with the predominant features of HUS in two-thirds of patients (5). Both autosomal recessive and autosomal dominant mode of inheritance have been recognized (5,6,9–12), with precipitating events such as pregnancy, virus-like disease, or sepsis being reported only in a minority of cases (13,14). Evidence that some of these cases responded, at least transiently, to plasma infusion or exchange led to the hypothesis that the genetic defect(s) associated with familial HUS/TTP may result in abnormalities in some plasma component(s) involved in the pathogenesis of the microangiopathic process. Thus, reduced serum levels of the third component (C3) of the complement system have been reported since 1974 in sporadic (15–18) and familial (19–21) forms of HUS. An inherited defect in C3 synthesis has been suggested to account for decreased C3 serum concentration (22), but much more convincing data are now available that low C3 in HUS may derive from either the lack of (21,23) or altered function of (24) factor H. In a very recent study, Warwicker and coworkers provided molecular evidence of the involvement of factor H in HUS (24). Indeed, they found that in three families with HUS, an area on chromosome 1q where factor H is mapped segregates with the disease. A deficiency in factor H—a plasma protein that inhibits the formation and accelerates the decay of the alternative pathway enzyme (C3bBb) of complement activation (25–27)—may accelerate C3 tissue deposition and consumption (28). On the other hand, epidemiologic evidence of an association between decreased factor H and C3 bioavailability and familial HUS is missing. Furthermore, no information on the prevalence and on the predisposing role of factor H and C3 deficiency in familial forms presenting with the clinical signs of TTP is available so far.

Thus, the aim of present study was to formally investigate in a case-control design the relationship between C3 deficiency and familial cases of either HUS and TTP and to assess whether C3 defect may be related to an impaired factor H bioavailability.

Materials and Methods

Patients and Definitions

Thirty-five cases of familial HUS/TTP were identified among 10 families through the database of the Italian Registry of Recurrent and Familial HUS/TTP, a network of 50 units of Hematology and Nephrology, established on 1995, under the coordination of the Clinical Research Center for Rare Diseases "Aldo & Cele Daccò" (Ranica, Italy). The following criteria were given to guide patient selection.

Diagnosis of HUS and TTP. HUS or TTP was diagnosed in all cases reported to have one or more episodes of microangiopathic hemolytic anemia and thrombocytopenia defined on the basis of hematocrit (Ht) <30%, hemoglobin (Hb) <10 mg/dl, serum lactate dehydrogenase (LDH) >460 U/L, undetectable haptoglobin, fragmented erythrocytes in the peripheral blood smear, and platelet count <150,000/μl.

Differential Diagnosis between HUS and TTP. Because of their frequently overlapping clinical and laboratory features, differential diagnosis between HUS and TTP is often uncertain and controversial. For the purposes of the study, the prevalence of signs of renal or neurologic involvement was taken as criteria to differentiate HUS and TTP, respectively (1,3,4). Cases presenting with the features of either HUS or TTP in different episodes in the same patients or in different patients within the same family were defined as HUS/TTP.

Diagnosis of Familial HUS and TTP. HUS and TTP were defined as familial when at least two members of the same family were affected by the disease at least 6 mo apart, and exposure to a common environmental triggering agent (in particular a verotoxin-producing strain of *Escherichia coli*) could be reasonably excluded.

Diagnosis of Relapsing HUS and TTP. Relapsing HUS or TTP was diagnosed when one or more episodes of the disease were diagnosed in the same subject after complete and persistent (for at least 2 wk off any kind of specific therapy, in particular plasma infusion or exchange) remission of any sign of microangiopathic hemolysis.

Diagnosis of Hypocomplementemia. Plasma levels of the third and/or fourth (C4) complement component below the lower limit of normal ranges (defined as mean ± 2 SD) of the laboratories of the "Ospedali Riuniti, Azienda Ospedaliera di Bergamo" (*i.e.*, <83 mg/dl for C3 and <15 mg/dl for C4) were taken to indicate hypocomplementemia.

Study Design

Of the 35 cases identified in 10 families through the database of the Registry, 16 (9 males, 7 females) were alive at the time of the study and 19 had died because of the disease. The 16 alive patients came from nine families, and both affected subjects in the remaining family (family 15) had died before our investigation. All relevant information in all of the 35 identified cases were recorded through the database of the Registry and by reviewing the patients' charts. All 16 patients who were alive at the time of the study and all of their available relatives (n = 63, 27 males, 36 females) were given detailed information about the purpose and design of the project and provided informed consent to enter the study according to the Declaration of Helsinki guidelines. Patients and their relatives were therefore referred to the Clinical Research Center where a detailed history was recorded and a blood sample was collected from all subjects and processed for the laboratory tests listed below. For the purposes of the study, all of the subjects who had one or more episodes of the disease were referred as "cases" and their family members who never had the disease as "case-relatives." All cases except one were studied at disease remission. The case with clinical signs of active disease was not included in the data analysis.

On each occasion, a case and his or her case-relatives were referred to the Clinical Research Center, and one or two age- and gender-matched "controls" (n = 25, 11 males, 14 females) and all of their available relatives (referred to as "control-relatives;" n = 56, 29 males, 27 females) were simultaneously studied with their blood samples collected and processed under the same experimental conditions described for cases and their relatives.

Laboratory Evaluations

In addition to the third and fourth complement fractions, markers of disease activity (*i.e.*, serum LDH, haptoglobin concentration, and platelet count), renal dysfunction (*i.e.*, serum creatinine and urea concentration), and concomitant systemic diseases (including anti-double-stranded-DNA antibodies titer, lupus erythematosus phenomenon, lupus anticoagulant, anticardiolipin antibodies, serum cryoglobulin concentration) and routine biochemicals (complete blood cell count, hematocrit, hemoglobin concentration, serum proteins and electrolytes, uric acid, blood glucose, serum lipids, and urinalysis) were evaluated in all cases and controls referred to the Clinical Research Center. Factor H and its different isoforms were also evaluated in all affected families and in 17 controls. The biologic samples from all cases, case-controls, and corresponding case- and control-relatives were collected on the same occasion and processed in the same experimental setting.

C3 and C4 were quantified by kinetic nephelometric measurements. Anticardiolipin antibodies titer was quantified by an enzyme-linked immunosorbent assay method. Lupus anticoagulant was measured by the Russell's viper venom test and kaolin clotting time test. Factor H was quantified by a radial immunodiffusion assay using a sheep polyclonal antihuman factor H antiserum (The Binding Site, Birmingham, United Kingdom). In addition to factor H, a factor H-like protein (FHL-1), and various shorter proteins, factor H-related proteins (FHR) have been identified in human plasma (25,29). FHL-1 is derived from the factor H gene by alternative splicing and displays complement regulatory activities (25). FHR, which differ considerably from factor H, are likely transcribed from distinct genes and have none of the known activities of factor H (25). To search for possible qualitative and quantitative defects of the different factor H molecules, Western blot analysis of factor H, factor H-like, and factor H-related proteins in serum samples was also performed. Briefly, serum (1.5 μl) was separated by 10 or 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to Laemmli (30), using prestained bench markers (Life Technologies) as standards. Proteins were electroblotted to nitrocellulose by semidry blotting (31). Membranes were blocked for 30 min using 5% (wt/vol) dried milk in phosphate-buffered saline (PBS). Incubations with polyclonal goat anti-factor H antiserum (Calbiochem, diluted 1:1000) or anti-FHL-1 (*rabbit anti-SCR1–4 antiserum, which does not detect FHR-1* and FHR-2 proteins, dilution 1:1000) (29) were performed at 4°C overnight. After washing in PBS 5 times, membranes were incubated with peroxidase-conjugated rabbit anti-goat or swine anti-rabbit antibody (Dako, Hamburg, Germany), respectively, for 2 to 3 h. Protein bands were visualized by the addition of 0.3% (wt/vol) 4-chloro-1-naphtol in 10% (vol/vol) methanol in PBS. In family 24, which showed abnormal high molecular weight bands that reacted with anti-factor H antiserum, densitometric analysis was performed with a computer-based digital imaging processing. Autoradiograph of the gel was acquired using a digitizing board. High molecular weight bands (Figure 6A, black arrow) and normal factor H band (open arrow) were resolved into a series of peaks and areas under the peaks calculated by specific function of the software Image 1.60 (National Institutes of Health Bethesda, MD). The corresponding areas were computed, and the ratio of high molecular weight band/normal factor H band was calculated.

To calculate cofactor activity of factor H, in families 24 and 29, human C3b (200 μg; Advanced Research Technologies, San Diego, CA) was labeled with ^{125}I using the iodogen technique (32). The cofactor assay was performed as described (29). Briefly, serum was diluted 1:200 in Veronal-buffered saline, radiolabeled C3b was added, and the mixture was incubated at 37°C for 2 h. Serum from the individual patients, from the healthy family members, and from normal control subjects were treated identically. This treatment results in cleavage of the 112-kD α-chain of C3b into a 69-kD and a 43-kD fragment, leaving the 75-kD β-chain unaffected. After incubation, the mixture was reduced and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, gels were fixed for 10 min in 10% acetic acid, dried, and autoradiographed. Human ^{125}I C3b was used as a control. The cofactor activity of the samples was quantified by counting the radioactivity of the proteolytic α-43 band and normalizing to the radioactivity of the β-chain of the sample. All of the other tests were performed by routine laboratory procedures.

Statistical Analyses

Personal, clinical, and laboratory data were recorded on a uniform data extraction form (Registration Form). Database management was performed using FileMakerPro software, version 2.1 (Claris Corp., Santa Clara, CA). Dichotomous baseline characteristics were compared by χ^2 test or Fisher exact test, as appropriate, and continuous baseline characteristics by Wilcoxon rank sum test. Univariate and multivariate analyses were carried out by logistic regression model. Correlation analysis between continuous variables and dichotomous variables was carried out using point-biserial correlation coefficient (rpb) (33). Statistical significance was set at 0.05 (two-tailed).

Results

Distribution and outcome of the different forms of the disease in all 35 cases identified through the database of the Registry are summarized in Table 1. Of note, one-third of cases were adults with TTP, HUS, or HUS/TTP on different occasions. All children had HUS. One or more disease relapses were reported in 18 (51%) cases. Nineteen patients (one with TTP and 18 with HUS) had died before the study was conducted (54.3%); of these, one child died after receiving a kidney transplant. Among the 16 survivors, six were on chronic dialysis (37.5%). Of these, two had received a kidney transplant that failed because of disease recurrence. Two had neurologic sequelae. Conditions potentially predisposing to disease onset were recognized in 19 cases (54.3%), including an upper respiratory tract infection (n = 14), pregnancy (n = 4), and consumption of birth control pills (n = 1).

Consanguinity was observed in two families. In one family (family 5), the parents were first cousins and had six children,

Table 1. Prevalence and outcome of the different cases of familial HUS and TTP recorded in the database of the Italian Registry of Recurrent and Familial HUS/TTP[a]

Category	HUS	HUS/TTP	TTP	Total
Families	7	2	1	10
Patients	29	4	2	35
adults	6	4	2	12
children	23	0	0	23
Relapses	14	4	0	18
Deaths	18	0	1	19

[a] HUS, hemolytic uremic syndrome; TTP, thrombotic thrombocytopenic purpura.

four of whom developed HUS during infancy (three of them died from acute renal failure, and the fourth had several recurrences and is now well at the age of 5 yr). In the other family (family 29, $n = 10$, from Israel) described in detail elsewhere (20), composed of offspring of several interrelated families with a high rate of consanguinity, 10 infants manifested HUS early in life and eight died. The two survivors suffered several recurrences until they entered chronic dialysis. Pedigrees of the 10 families are shown in Figures 1, 3, and 5. Pattern of inheritance of HUS and TTP could not be unequivocally deduced because all families had only one generation affected. In family 4, two cases of death for acute renal failure were reported, without a definitive diagnosis. In the same family, history of renal disease was reported in two other relatives, again without specific diagnosis. These four subjects are indicated as probably affected in Figure 1.

At the time of study evaluation, one of the 16 alive patients had clinical signs of disease activity and was not included in final analysis. The latter patient, with HUS, was from family 19, and his brother with diagnosis of TTP, in remission, was included in the study. Two other patients, who presented only mild and isolated increases in serum LDH concentration with-

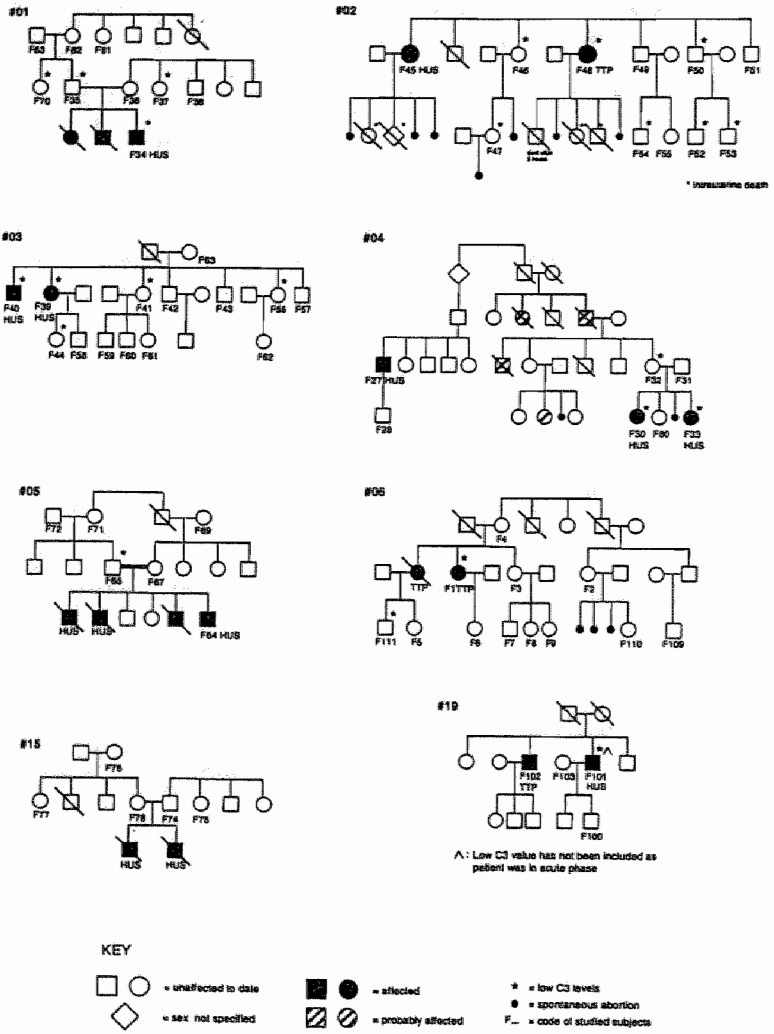


Figure 1. Pedigree of families 1, 2, 3, 4, 5, 6, 15, and 19 (*e.g.*, family 1 is indicated as #01). Individuals with low C3 serum concentrations are identified with an asterisk.

out any other laboratory or clinical signs of disease activity, were considered in remission and were included in the analysis. Thus, 15 patients (cases) entered the study. All of these patients were in stable remission, the time from the last clinical episode ranging from 5 mo to 15 yr. Main parameters in cases, matched controls, and case- and control- relatives referred to the Clinical Research Center are given in Table 2. By the Wilks–Shapiro test, all variables were found to be normally distributed, with the exception of serum creatinine, LDH, and C3 levels, only in cases. Median (range) values of these parameters in cases were as follows: serum creatinine: 2 (0.7 to 10.5) mg/dl; LDH: 385 (226 to 883) U/L; C3: 68 (10 to 99) mg/dl.

Seventy-three percent of cases compared with 16% of controls ($P < 0.001$), and 24% of case-relatives compared with 5% of control-relatives ($P = 0.005$) had decreased C3 serum levels (Figure 2). When families were analyzed separately (see pedigrees in Figures 1, 3A, and 5A), we found that in five of the nine families (families 1, 3, 6, 24, and 29) all cases had low C3 levels. In families 2 and 4, low C3 was found in one of two and in two of three cases, respectively. Of interest in family 2, a high incidence of low C3 was also observed in case-relatives, suggesting that low C3 strongly segregates to this family. In family 5, the only evaluable case (F64) had normal C3 serum concentration, whereas low C3 was found in his father. In only one family (family 19), C3 serum concentration was normal both in the case (F102) and in case-relatives (Figure 1).

Mean serum C3 concentration was significantly lower in cases compared with all of the other study groups (Table 2). Cases also had significantly lower hemoglobin and hematocrit, and higher serum creatinine and LDH compared with controls. Platelet count, serum C4 (Table 2 and Figure 2), anticardiolipin antibodies, lupus anticoagulant, as well as all of the markers of systemic disease not reported in the table, were within the normal ranges in all subgroups. Of note, no significant correlation could be demonstrated between serum C3 concentration and serum LDH levels either in affected families or in the overall study population. Actually, in case F64 with the highest LDH value, serum C3 concentration was normal (LDH: 883 U/L, C3: 84 mg/dl), whereas the other case, F34, with higher than normal LDH, had low C3 serum concentration (LDH: 520 U/L, C3: 69 mg/dl).

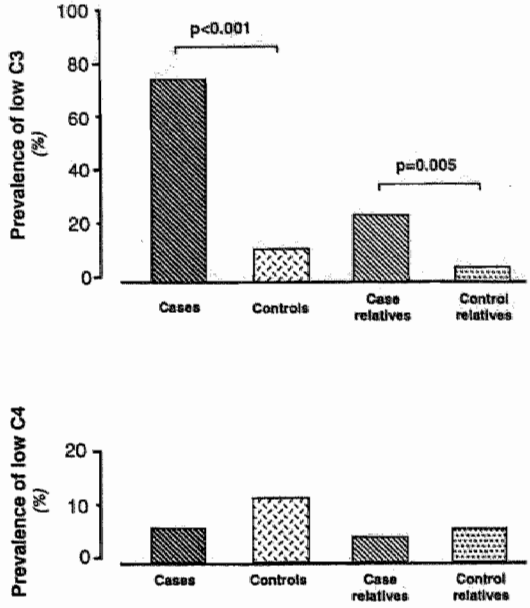


Figure 2. Prevalence of low C3 and C4 concentrations among cases ($n = 15$), controls ($n = 25$), case-relatives ($n = 63$), and control-relatives ($n = 56$) referred to the Clinical Research Center "Aldo e Cele Daccò."

Table 2. Main clinical and laboratory parameters in all subjects referred to the Clinical Research Center "Aldo Cele Daccò"[a]

Parameter	Cases ($n = 15$)	Controls ($n = 25$)	Case-Relatives ($n = 63$)	Control-Relatives ($n = 56$)
Age (yr)	26.6 ± 17.9[b]	34.7 ± 13.5[b]	36.9 ± 20.9[b]	45.8 ± 16.5
Gender (M/F)	8/7	11/14	27/36	29/27
Serum creatinine (mg/dl)	3.8 ± 3.9[c]	0.9 ± 0.1	0.8 ± 0.2	0.9 ± 0.2
Hemoglobin (mg/dl)	11.4 ± 2.5[c]	14.0 ± 1.1	13.7 ± 1.3	14.2 ± 1.5
Hematocrit (%)	34.3 ± 7.0[c]	42.3 ± 3.5	40.6 ± 3.2[b]	42.4 ± 4.2
Platelet count ($10^3/\mu l$)	221 ± 60	220 ± 51[d]	251 ± 70[b]	213 ± 49
LDH (U/L)	384.5 ± 180.6	277.4 ± 57.2[d]	340.0 ± 90.5[b]	297.8 ± 57.9
ACA (U/ml)	9.7 ± 6.9	9.9 ± 5.8	10.1 ± 8.1	11.6 ± 6.9
C3 (mg/dl)	67.0 ± 26.7[c]	97.0 ± 19.5	100.4 ± 25.4	100.2 ± 18.0
C4 (mg/dl)	25.7 ± 11.8	21.9 ± 6.8	25.3 ± 9.5[b]	22.1 ± 7.2

[a] Data are mean ± SD. LDH, lactate dehydrogenase; ACA, anticardiolipin antibodies.
[b] $P < 0.05$ *versus* control-relatives.
[c] $P < 0.05$ *versus* all of the others.
[d] $P < 0.05$ *versus* case-relatives.
[e] $P < 0.0005$ *versus* all of the others.

Table 3. Univariate correlation analysis between main clinical and laboratory parameters and familial HUS and TTP in the affected families and in the overall study population[a]

Parameter	*P* Value	
	Affected Families	All Subjects
Age (yr)	0.09	0.02
Gender (M/F)	0.47	0.62
Serum creatinine (mg/dl)	0.02	0.003
Hemoglobin (mg/dl)	0.0008	0.0001
Hematocrit (%)	0.002	0.0001
Platelet count ($10^3/\mu l$)	0.13	0.78
LDH (U/L)	0.23	0.02
ACA (U/ml)	0.83	0.61
C3 (mg/dl)	0.0007	0.0001
C4 (mg/dl)	0.88	0.36

[a] Abbreviations as in Tables 1 and 2.

By univariate analysis (Table 3), lower levels of C3, hemoglobin, and hematocrit, and higher levels of serum creatinine were significantly associated with HUS or TTP either when the analysis was performed among affected families or in the overall study population. In the overall study population, a significant association was also found with age and LDH. Multivariate logistic analysis accounting for the above risk factors showed that only C3 levels were significantly associated with HUS or TTP either among the affected families ($P = 0.02$) or the overall study population ($P = 0.01$). The relative risk of HUS or TTP associated with low C3 levels (C3 < 83 mg/dl) among the affected families and in the overall study population is shown in Table 4.

In all cases ($n = 4$) in whom repeated C3 measurements in disease remission were available, C3 levels were persistently lower than normal. Factor H concentration, measured by radial immunodiffusion, was abnormally low in one family with history of HUS (family 29) and high degree consanguinity. Factor H was severely depressed in the two affected subjects alive at the time of the study (Figure 3), F29 and F85. In two healthy relatives (F79 and F87), factor H levels were moderately below normal range; the other healthy relatives (F73, F84, and F86) had borderline values (Figure 3), suggesting a partial, heterozygous factor H deficiency. These data, together with the high degree consanguinity in this family, suggest a recessive pattern of inheritance for factor H defect. In the other families, serum factor H levels were within the normal range both in cases and case-relatives.

To search for possible abnormalities of serum levels and pattern of factor H and factor H-related proteins in families with HUS or TTP, sera from cases, their relatives, and controls were further analyzed by Western blotting. The results of the two cases and five healthy relatives from family 29 are shown in Figure 4. Western blot confirmed low levels of factor H in the serum of the two cases (F29 and F85, tracks 1 and 5), whereas no gross abnormality was detected in the serum of their healthy relatives (tracks 2, 3, 4, 6, and 7; track 8 represents serum from one healthy volunteer for comparison). In another family (family 24) with history of HUS, the two affected subjects (F106 and F108) who had normal factor H levels by radial immunodiffusion (Figure 5) showed abnormalities as identified by Western blotting. In the above patients (Figure 6A, tracks 5 and 6) and in their healthy father (track 2), additional high molecular weight bands were observed that reacted with anti-factor H antiserum and that were found with another pattern and different intensities in the control subject (track 1) and in the healthy mother (track 3) and sister (track 4). The nature of these bands of lower mobility is not clear. Conceivably, the cases inherited the factor H anomaly from their father. Densitometric analysis showed that the ratio of high molecular weight band/normal factor H band was higher in patients (F106: 0.42; F108: 0.19) and the healthy father (F104: 0.29) than in control subject (0.08). In the two cases, an additional band was observed with a ratio of 0.2 (F106) and 0.08 (F108) with respect to normal factor H band.

In Figure 6B, bands corresponding to the low molecular mass members of the factor H family, FHR-1α (37 kD) and FHR-1β (42 kD), are presented. Since FHL-1 band is masked by FHR-1β (they comigrate on Western blot) (25), FHL-1 pattern was analyzed with additional Western blots performed using a specific antibody anti-FHL-1 (not shown). For all proteins, similar expression levels and identical mobilities were identified in the serum of family 24 (Figure 6B) and family 29 (data not shown). No abnormalities in factor H and factor H-related proteins were found by Western blot analysis of sera from the other families.

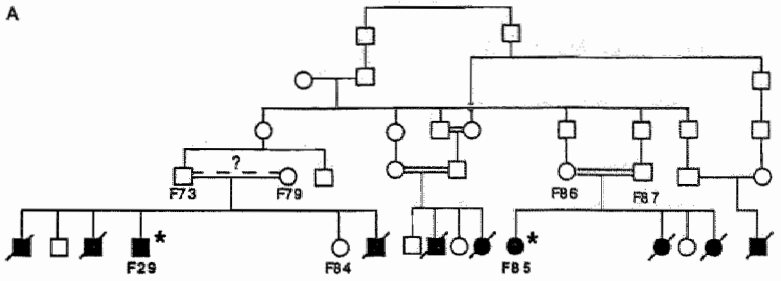
Altogether, factor H abnormalities (either in levels or Western blot pattern) were found in four of the 15 cases, compared with three of the 63 family members ($P = 0.02$) and none of

Table 4. Relative risk (95% confidence interval) of familial HUS and TTP associated with low C3 concentration within affected families and within the overall studied subjects

Parameter	Univariate Analysis		Multivariate Analysis	
	Affected Families	All Subjects	Affected Families	All Subjects
Relative risk	8.62	14.35	16.56	27.77
95% CI	2.39 to 31.10	4.20 to 49.00	1.66 to 162.39	2.44 to 314.19
P value	0.001	0.001	0.016	0.008

[a] CI, confidence interval.

A



B

	F 29	F73	F 79	F 84	F 85	F 86	F 87	(normal range)
C3 (mg/dl)	14	133	135	107	10	103	86	(83-177)
C4 (mg/dl)	42	42	49	24	40	34	36	(15-45)
Factor H (mg/l)	< 70	344	252	358	< 70	362	274	(367-619)

Figure 3. (A) Pedigree of family 29. Open symbols, unaffected to date; closed symbols, affected; *, low C3 levels; F (followed by a number), code of studied subjects. (B) Serum complement profile in family 29.

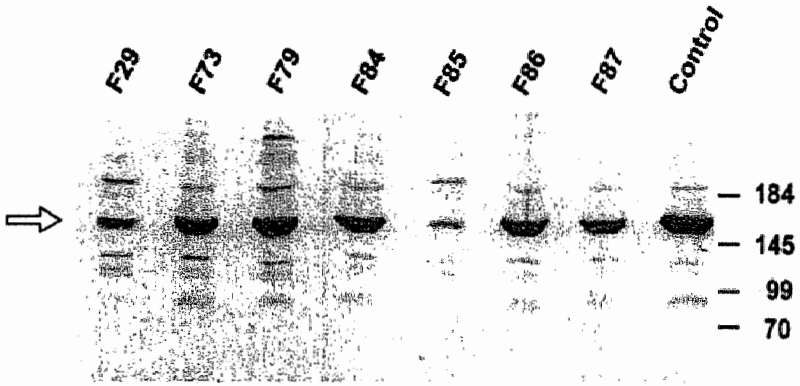


Figure 4. Western blot analysis of factor H in human serum (family 29). Sera were separated by 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the Western blot was developed using a polyclonal goat anti-factor H antiserum. Open arrow indicates factor H. F29, case (with inactive HUS); F73, F29 father; F79, F29 mother; F84, F29 sister (healthy); F85, case (with inactive HUS); F86, F85 mother; F87, F85 father. F86, F87, and F73 are first cousins.

the 17 healthy controls ($P = 0.04$). All cases with factor H abnormalities had low C3 serum concentration. Overall, factor H abnormalities were more frequent in the 15 cases than in the 80 subjects who never had the disease ($P < 0.02$). Within families, factor H abnormalities were significantly correlated with C3 reduction (rpb = -0.25; $P < 0.05$).

To further confirm the abnormalities of factor H in families 24 and 29 on a functional level, the cofactor activity of the case sera was compared with that of healthy relatives (Figure 7). For family 29, both cases showed a marked reduction in serum cofactor activity, as evidenced by the low intensity of the proteolytically cleaved α-chains (α-43). Under identical conditions, sera from both cases were rather inefficient in conversion of the C3b α-chain (Figure 7A). Serial dilution and densitometric analysis (data not shown) of the newly formed α-43 band showed that in both cases cofactor activity was approximately 10% of the values found in control sera. The rather low cofactor activity detected in the two cases of family

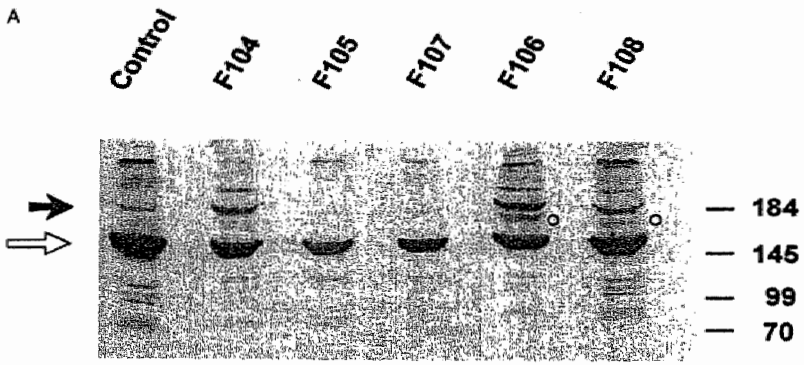
A

B

	F104	F105	F107	F106	F108	(normal range)
C3 (mg/dl)	130	97	96	61	61	(83-177)
C4 (mg/dl)	32	24	27	16	15	(15-45)
Factor H (mg/L)	835	546	562	711	569	(367-619)

Figure 5. (A) Pedigree of family 24. Symbols as in Figure 3. (B) Serum complement profile in family 24.

A



B

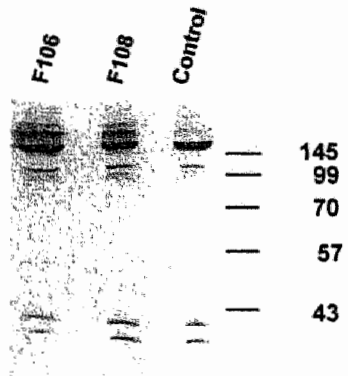


Figure 6. Western blot analysis of factor H and factor H-related proteins in human serum (family 24). Sera were separated by either 7% (A) or 10% (B) SDS-PAGE, and the Western blot was developed using a polyclonal goat anti-factor H antiserum. (A) F106 and F108, cases (with inactive HUS, brothers); F104, father; F105, mother; F106, sister. Open arrow indicates factor H. Closed arrow and circle indicate the abnormal high molecular weight bands. (B) 1 = factor H; 2 = factor H-related protein-1β; 3 = factor H-related protein-1α.

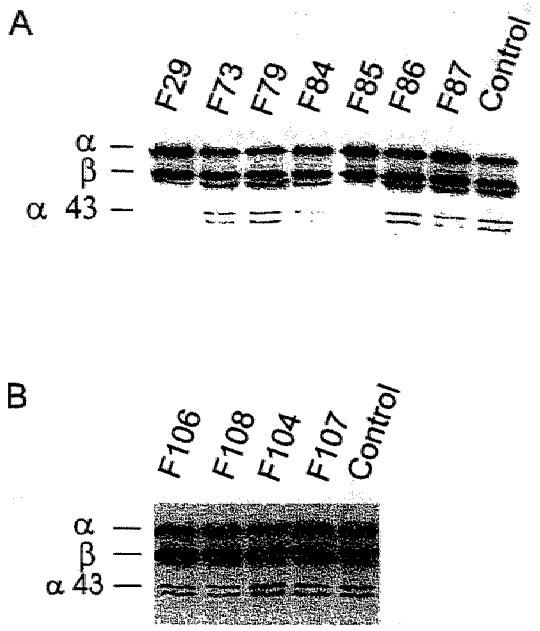


Figure 7. Cofactor activity of factor H in serum samples from family 29 (A) and family 24 (B). Radiolabeled C3b cleavage products were separated by SDS-PAGE under reducing conditions and autoradiographed. α and β represent the intact chains of C3b. Cofactor activity was calculated by densitometric analysis of the band corresponding to the α43 proteolytic product.

29 is in agreement with the low serum levels of factor H (Figure 3B), as also confirmed by Western blot analysis (Figure 4). A different result was observed in family 24. Sera obtained from both cases and healthy relatives showed similar activities that were all comparable to unrelated healthy controls (Figure 7B).

Discussion

The first finding of the present study was that approximately 75% of the 35 patients with familial HUS or TTP referred to the Registry died or had permanent neurologic or renal sequelae. Of note, the outcome was extremely poor also in the 29 patients presenting with the clinical features of HUS, of which 18 died at the time of the study. These data are in line with previous series (5,6) and provide further evidence of the extremely poor prognosis of this disease, which, regardless of the clinical presentation (*i.e.*, as HUS or TTP), is virtually unaffected by any form of treatment, including plasma manipulation, proven effective in most nonfamilial cases. Of interest, in two families the disease presented either as HUS or TTP, which, in line with previous reports (5,34,35), provides clear evidence of the close relationship between the two syndromes.

That the disease may be related to an inherited congenital abnormality is consistent with the finding that more than 50% of cases in the present series—compared with only 10 to 20% in nonfamilial forms (3)—had repeated recurrences either of TTP or HUS. That this genetic abnormality involves the complement system is suggested by levels of circulating C3, which were extremely low in familial cases compared with controls. Reduced C3 levels in cases and case-relatives, but not in controls and control-relatives, further indicate that the defect clusters in families. Evidence that low C3 concentration is strongly associated with the disease even more convincingly suggests the possibility of a close (possibly causal) relationship between decreased C3 and disease manifestation. On the other hand, in the present series, low C3 levels could not derive from consumption in a still-ongoing microangiopathic process, because no patient at the time of the study had any sign of acute disease, and only two had moderately increased LDH levels. Furthermore, failure to detect any correlation between C3 and serum LDH levels definitely ruled out the possibility that low C3 concentration merely reflected disease activity in our series.

Actually, low C3 levels are well known to accompany the acute phases either of typical (epidemic) and atypical (sporadic) HUS (15–18), and likely reflect C3 consumption in the microvasculature. Along this line are findings of granular C3 deposits in glomeruli and arterioles of HUS patients (1,18,36,37) and evidence of C3 breakdown products in HUS sera (15,16,18), which further document the activation of the complement system in the acute phase of the disease. Enhanced release of complement cleavage products (including C3a and C5a) consequent to uncontrolled complement activation may contribute to the microangiopathic process by stimulating neutrophil activation (38), phagocytic adhesion to vascular endothelium, or platelet aggregation, and by directly injuring the endothelium through enhanced production of the membrane attack complex, the final multimolecular unit of the complement C5b-9 (39).

Two complement pathways can generate C3-activating enzymes: the classical convertase generated by the sequential reaction of C1, C4, and C2, and the alternative pathway convertase (40). Activation of classical and alternative complement pathway—possibly triggered by circulating immune complexes (41) and damaged erythrocytes (42), respectively—is well documented in acute HUS (16,18), but consistently subsides with remission of the disease. On the contrary, in our series, serum C3 levels were consistently and remarkably depressed in cases compared with controls, even during remission of the disease. Even more interesting, low C3 levels were also found in the relatives of our patients who had never suffered from HUS or TTP in the past and had no sign of the disease at the time of the study. Furthermore, either in cases or in case-relatives depressed C3 values were not paralleled by similar changes in C4 levels, which definitely ruled out the possibility that classical pathway activation accounted for hypocomplementemia in our series. Alternative explanations for reduced C3 bioavailability in familial HUS and TTP must therefore be provided.

Previous studies found low C3—but, notably normal C4—levels in occasional families affected by one or more cases of HUS (19–21,23), which at least in one case were found to

persist even during recovery from the disease (23). In this patient and in a healthy brother as well, low C3 levels were accompanied by very low levels of factor H, a regulatory protein that inhibits the complement activation through the alternative pathway. Finding that the parents, who were first cousins, had half-normal levels of factor H, convincingly indicated that the defect was inherited. Similar findings were then reported in another family (21). The above reports raised the intriguing possibility that low C3 in the setting of familial HUS may depend on an inherited deficiency of factor H. This possibility is in line with the evidence provided by Warwicker and coworkers in three large families with HUS, that an area on chromosome 1q, where factor H gene is mapped, segregates with the disease (24). All subjects in the three families had normal serum factor H levels; however, affected members and obligate carriers within one family were found, by mutation analysis, to have a point mutation in factor H causing an arginine to glycine change (24). This mutation is likely to alter structure and hence function of factor H protein without modifying its circulating levels. Another mutation comprising a deletion in factor H gene, which—through a frameshift and subsequent premature termination codon—led to a 50% reduction in serum factor H levels, has been described even in a subject with relapsing HUS (24).

By radial immunodiffusion and Western blot analysis, we identified in our series two affected subjects of one family who had very low circulating factor H levels, and moderately low levels were found in two healthy relatives. In these patients, cofactor activity of factor H, measured as the capacity to degrade C3b, was also reduced.

In the other families, serum factor H concentration was normal. However, finding normal serum levels does not necessarily exclude an underlying biochemical abnormality in circulating factor H, possibly related to mutations in the gene that leads to the synthesis of an abnormal protein (24). In this regard, in another family of our series, the two affected members, and the healthy father who had normal serum concentrations of factor H by radial immunodiffusion, showed on Western blot additional bands of higher molecular weight that were not found in any control. The nature of these bands is not clear, and molecules related to factor H with such large size having not yet been identified. If one were to guess, the bands may represent dimeric forms of factor H. At variance, no differences were found in serum levels and patterns of FHL-1 and FHR proteins (25). *In vitro*, serum from the two cases of this family showed normal factor H activity, which was consistent with normal factor H concentration found by radial immunodiffusion. Similar results have been obtained by Warwicker and coworkers in their three families (24). Actually, factor H has several biologic activities: (*1*) It prevents the formation of C3bBb complex and accelerates the dissociation of Bb from the C3 convertase. (*2*) It acts as a cofactor for factor I, which degrades C3b (25). (*3*) It distinguishes between activator and nonactivator surfaces (25). Other less well defined functions of factor H have been suggested by the presence in factor H protein of at least two heparin-binding sites that could facilitate interaction with extracellular matrix (25). Thus, it is hard to disclose by *in vitro* tests how a given molecular alteration of factor H actually affects its complex biologic activity *in vivo*.

Of note, a significant association was found in families between factor H abnormalities and low C3 levels, which supports the hypothesis that low C3 in the setting of familial HUS/TTP may depend on an inherited deficiency of factor H.

Genetic deficiency of factor H has been described in a relatively small number of families (24,26–28). Patients with homozygous factor H deficiency suffer recurrent bacterial infections (43), vasculitis, and/or glomerulonephritis (21,44). In a recent article (28), Ault and coworkers described a 13-mo-old boy who presented with hypocomplementemic hypertensive renal disease. Renal biopsy showed changes consistent with membranous proliferative glomerulonephritis and segmental C3 deposition in capillary loops. He had decreased serum levels of C3 and factor H was undetectable. Sequence analysis revealed two different point mutations in factor H gene, one on each allele.

It is also possible that genetic defects in other complement regulatory proteins (DAF, CR1, CR2, C4bp) might have a role in determining low C3 in familial HUS and TTP. Data (45) that the above genes map on the same region of chromosome 1q as factor H would support this hypothesis.

Although it seems clear that familial HUS and TTP are related to an inherited congenital defect, the cause of the syndrome is probably multifactorial and the inherited complement defects here documented may just represent a predisposing condition that increases the risk of the disease in combination with other intercurrent environmental or acquired factors. Thus, in a family with hereditary hypocomplementemia reported to our Registry (not shown), only one of the six members with low C3 levels developed HUS, apparently after a flulike disease.

But which is the sequence of events that may lead to disease manifestation in subjects with inherited congenital C3/factor H abnormalities? We suggest that an intercurrent exposure to agents potentially toxic to the vascular endothelium—such as certain viruses, bacteria, toxins, immunocomplexes, and cytotoxic drugs (1,2)—may initiate a local intravascular thrombosis, which promotes C3bBb convertase formation and complement deposition within capillary vessels (42,46,47). In normal conditions, however, factor H by modulating C3bBb activity (25–27) may effectively limit complement deposition and further extension of the process. On the contrary, when the bioavailability and/or the activity of factor H is congenitally defective, C3bBb convertase formation and complement deposition may become uncontrolled, with further extension of the microangiopathic process up to full manifestation of the disease.

Whatever the pathogenetic mechanism(s) accounting for decreased C3 levels in familial HUS and TTP, evidence that low C3 clusters in families affected by HUS and TTP and that, among family members, low *versus* normal C3 is associated with a more than 16 times greater risk of the disease, may have two major clinical implications. First, quantification of serum C3 concentration in cases at remission and in their relatives could be a useful tool to investigate the possibility of a familial

form of the disease. Second, when the diagnosis of familial HUS and TTP is established, serum C3 concentration could be quantified in all of the family members to identify the subjects at increased risk.

In conclusion, in the present study we found that an alteration of the third complement component clusters in families affected by familial HUS and TTP. Reduced C3 serum levels may reflect enhanced C3 consumption secondary to a genetically determined factor H deficiency and may predispose to microvascular thrombosis in familial HUS and TTP: Its demonstration may help identify subjects at risk within families who may benefit the most from genetic counseling and careful monitoring.

Appendix 1: Organization of the Italian Registry for Recurrent and Familial HUS/TTP

Coordinators

P. Ruggenenti, MD, M. Noris, Chem. Pharm. D., G. Remuzzi, MD (Clinical Research for Rare Diseases "Aldo e Cele Daccò," Ranica).

Investigators

F. Casucci, MD, F. Cazzato, MD (Division of Nephrology, "Miulli" Hospital, Acquaviva delle Fonti, Bari); **M. Dugo, MD** (Division of Nephrology and Dialysis, "S. Giacomo" Hospital, Castelfranco Veneto, Treviso); **C. Cappelletti, MD, C. Ceccarelli, MD** (Division of Internal Medicine and Division of Nephrology, "S. Giovanni di Dio" Hospital, Firenze); **G. C. Barbano, MD** (Division of Pediatric Nephrology, "G. Gaslini" Institute, Genova); **A. Edefonti, MD** (Division of Pediatric Dialysis, "De Marchi" Pediatric Clinic, Milano); **E. Rossi, MD** (Blood Transfusion Center, "L. Sacco" Hospital, Milano); **G. B. Haycock, MD** (Pediatric Renal Unit, Guy's Hospital, London, United Kingdom); **A. Indovina, MD** (Bone Marrow Transplantion Unit, "V. Cervello" Hospital, Palermo); **B. Vasile, MD, E. Daina, MD, S. Gamba, Research Nurse, A. Schieppati, MD** (Information Center for Rare Diseases, Ranica, Bergamo); **C. Zoccali, MD, F. Mallamaci, MD** (Division of Nephrology and Dialysis, Hospital of Reggio Calabria); **T. Cicchetti, MD, G. Putortì, MD** (Division of Nephrology and Dialysis, "N. Giannettasio" Hospital, Rossano Calabro); **D. Landau, MD** (Pediatric Nephrology, Soroka Medical Center, Beer-Sheba, Israel); **O. Amatruda, MD** (Division of Nephrology, "Fondazione Macchi" Hospital, Varese).

Contributing Correspondence

R. Bellantuono, MD, T. De Palo, MD (Division of Nephrology and Dialysis, "Giovanni XXIII" Pediatric Hospital, Bari); **T. Barbui, MD, M. Galli, MD,** (Division of Hematology,"Ospedali Riuniti Azienda Ospedaliera," Bergamo); **S. Bassi, MD** (Division of Nephrology and Dialysis, Umberto I Hospital, Brescia); **R. Wens, MD** (CHU Brugman, Bruxells, Belgium); **M. G. Caletti, MD** (Servicio de Nefrologia, Hospital Garrahan, Buenos Aires, Argentina); **E. Grandone, MD, F. Aucela, MD** (Atherosclerosis and Thrombosis Research Laboratory and Division of Nephrology and Dialysis, "Casa Sollievo della Sofferenza S. Giovanni Rotondo" Hospital, Foggia); **E. Pogliani, MD, D. Belotti, Biol. Sci. D.** (Divison of Hematology and Transfusion Center, "San Gerardo" Hospital, Monza); **V. Toschi, MD** (Transfusional Center, "San Carlo Borromeo" Hospital, Milano); **P. Prandoni, MD** (Division of Medicine, Hospital of Padova); **R. Marceno, MD** (Bone Marrow Transplantion Unit, "V. Cervello" Hospital, Palermo); **T. Cocchi, MD, M. Sassi, MD** (Blood Transfusion Center, Hospital of Parma); **C. Porta, MD** (Division of Medicine, IRCCS Policlinico "San Matteo," Pavia); **G. F. Rizzoni, MD, A. Gianviti, MD** (Division of Nephrology and Dialysis, "Bambino Gesù" Pediatric Hospital, Roma); **A. Pinto, MD** (Division of Nephrology and Dialysis, "S. Giovanni di Dio e Ruggi D'Aragona," Salerno); **M. Sanna, MD** (Division of Medical Pathology, Hospital of Sassari); **R. Coppo, MD, A. Amore, MD** (Division of Nephrology and Dialysis, "Regina Margherita" Pediatric Hospital, Torino); **A. Khaled, MD** (Division of Nephrology, "S. Chiara" Hospital, Trento); **A. Cao, MD** (Institute of Clinic and Biology of Evolutive Age, Cagliari); **F. Della Grotta, MD** (Division of Nephrology, Hospital of Anzio).

Laboratory Analysis

L. Robba, MD, A. Vernocchi, MD, A. Crippa, MD (Division of Laboratory Analysis, "Ospedali Riuniti, Azienda Ospedaliera," Bergamo); **F. Gaspari, Chem. D.** (Clinical Research for Rare Diseases "Aldo e Cele Daccò," Ranica, Bergamo).

Biochemical Studies

M. Ghilardi, Biol. Sci. D., D. Macconi, Biol. Sci. D., M. Galbusera, Biol. Sci. D., C. Rossi, Chemist, S. Orisio, Biol. Sci. D., J. Caprioli, Biol. Sci. D. ("Mario Negri" Institute for Pharmacological Research, Bergamo); **P. F. Zipfel, Ph. D., C. Skerka, Ph. D., Eva Kampen, Chemist** (Bernard Nocht Institute für Tropenmedizin, Hamburg).

Statistical Analysis

A. Perna, Stat. Sci. D. (Clinical Research for Rare Diseases "Aldo e Cele Daccò," Ranica).

Acknowledgments

This study was supported by the Italian Telethon Grant E359 and by a Baxter extramural grant. Dr. Caprioli is a fellow of "Associazione per la Ricerca sulle Malattie Rare" (Bergamo, Italy). Dr. M. Ghilardi, who is a fellow of Rotary Club Bergamo Nord, performed radial immunodiffusion assay of factor H. Drs. L. Robba, A. Crippa, and A. Vernocchi measured the relevant biochemical parameters, including the serum complement levels. We thank Eva Kampen for expert technical assistance.

References

1. Remuzzi G, Ruggenenti P, Bertani T: Thrombotic microangiopathy. In: *Renal Pathology With Clinical and Functional Correla-*

tions, edited by Tisher CC, Brenner BM, 2nd Ed., Philadelphia, Lippincott, 1994, pp 1154–1184
2. Remuzzi G, Ruggenenti P: The hemolytic uremic syndrome. *Kidney Int* 47: 2–19, 1995
3. Ruggenenti P, Remuzzi G: The pathophysiology and management of thrombotic thrombocytopenic purpura. *Eur J Haematol* 56: 191–207, 1996
4. Remuzzi G: HUS and TTP: Variable expression of a single entity. *Kidney Int* 32: 292–308, 1987
5. Kaplan BS, Kaplan P: Hemolytic uremic syndrome in families. In: *Hemolytic Uremic Syndrome and Thrombotic Thrombocytopenic Purpura*, edited by Kaplan BS, Trompeter RS, Moake JL, New York, Marcel Dekker, 1992, pp 213–225
6. Berns JS, Kaplan BS, Mackow RC, Hefter LG: Inherited hemolytic uremic syndrome in adults. *Am J Kidney Dis* 19: 331–334, 1992
7. Fitzpatrick MM, Dillon MJ, Barratt TM, Trompeter RS: Atypical hemolytic uremic syndrome. In: *Hemolytic Uremic Syndrome and Thrombotic Thrombocytopenic Purpura*, edited by Kaplan BS, Trompeter RS, Moake JL, New York, Marcel Dekker, 1992, pp 163–178
8. Sheth KJ, Gill JC, Leichter HE, Havens PL, Hunter JB: Increased incidence of HLA-B40 group antigens in children with hemolytic-uremic syndrome. *Nephron* 68: 433–436, 1994
9. Kirchner KA, Smith RM, Gockerman JP, Luke RG: Hereditary thrombotic thrombocytopenic purpura: Microangiopathic hemolytic anemia, thrombocytopenia, and renal insufficiency occurring in consecutive generations. *Nephron* 30: 28–30, 1982
10. Bergstein J, Michael A Jr, Kjellstrand C, Simmons R, Najarian J: Hemolytic-uremic syndrome in adult sisters. *Transplantation* 17: 487–489, 1974
11. Pirson Y, Lefebvre C, Arnout C, de Strihou CY: Hemolytic uremic syndrome in three adult siblings: A familial study and evolution. *Clin Nephrol* 28: 250–255, 1997
12. Carreras L, Romero R, Requesens C, Oliver AJ, Carrera M, Clavo M, Alsina J: Familial hypocomplementemic hemolytic uremic syndrome with HLA-A3, B7 haplotype. *JAMA* 245: 602–604, 1981
13. Feest TG, Hamilton W, Imong S: Severe ocular and pulmonary manifestations of familial haemolytic uremic syndrome: Possible benefit of bilateral nephrectomy. Proceedings of the 23rd Congress of EDTA-ERA-Budapest, 1986
14. Bukowski RM: Thrombotic thrombocytopenic purpura: A review. In: *Progress in Hemostasis and Thrombosis*, edited by Spaet TH, New York, Grune & Stratton, 1982, p 287
15. Stuhlinger W, Kourilsky O, Kanfer A, Sraer JD: Haemolytic-uraemic syndrome: Evidence for intravascular C3 activation. *Lancet* ii: 788, 1974
16. Monnens L, Molenaar J, Lambert PH, Proesmans W, van Munster P: The complement system in hemolytic-uremic syndrome in childhood. *Clin Nephrol* 13: 168–171, 1980
17. Robson WLM, Leung AKC, Fick GH, McKenna AI: Hypocomplementemia and leukocytosis in diarrhea-associated hemolytic uremic syndrome. *Nephron* 62: 296–299, 1992
18. Kim Y, Miller K, Michael AF: Breakdown products of C3 and factor B in hemolytic-uremic syndrome. *J Lab Clin Med* 89: 845–850, 1977
19. Zachwieja J, Strzykala K, Golda W, Maciejewski J: Familial, recurrent hemolytic-uremic syndrome with hypocomplementaemia. *Pediatr Nephrol* 6: 221–222, 1992
20. Landau D, Ohali M, Shalev H, Schlezinger M, Maor E, Carmi R: Immune-mediated pathogenesis of familial hypocomplementemic hemolytic uremic syndrome (HUS) [Abstract]. *J Am Soc Nephrol* 7: 1616, 1996
21. Pichette V, Quérin S, Schürch W, Brun G, Lehner-Netsch G, Delaghe J-M: Familial hemolytic-uremic syndrome and homozygous factor H deficiency. *Am J Kidney Dis* 26: 936–941, 1994
22. Roodhooft AM, McLean RH, Elst E, Van Acker KJ: Recurrent hemolytic uremic syndrome and acquired hypomorphic variant of the third component of complement. *Pediatr Nephrol* 4: 597–599, 1990
23. Thompson RA, Winterborn MH: Hypocomplementaemia due to a genetic deficiency of b1H globulin. *Clin Exp Immunol* 46: 110–119, 1981
24. Warwicker P, Goodship THJ, Donne RL, Pirson Y, Nicholls A, Ward RM, Turnpenny P, Goodship JA: Genetic studies into inherited and sporadic hemolytic uremic syndrome. *Kidney Int* 53: 836–844, 1998
25. Zipfel PF, Skerka C: Complement factor H and related proteins: An expanding family of complement-regulatory proteins? *Immunol Today* 15: 121–126, 1994
26. Nilsson UR, Müller-Eberhard HJ: Isolation of b_{1F}-globulin from human serum and its characterization as the fifth component of complement. *J Exp Med* 122: 277–298, 1965
27. Vik DP, Munor-Canoves P, Chaplin DD, Tack BF: Factor H. *Curr Top Microbiol* 153: 147–160, 1989
28. Ault BH, Schmidt BZ, Fowler NL, Kashtan CE, Ahmed AE, Vogt BA, Colten HR: Human factor H deficiency: Mutations in framework cysteine residues and block in H protein secretion and intracellular catabolism. *J Biol Chem* 272: 25168–25175, 1997
29. Kühn S, Skerka C, Zipfel PF: Mapping of the complement regulatory domains in the human factor H-like protein 1 and factor H. *J Immunol* 155: 5663–5670, 1995
30. Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press, 1989
31. Kyhse-Andersen J: Electroblotting of multiple gels: A simple apparatus without buffer tank for transfer of proteins from polyacrylamine gels. *J Biochem Biophys Methods* 10: 203–209, 1984
32. Greenwood FC, Hunter WM, Glover JS: The preparation of ^{125}I-labeled human growth hormone of high specific activity. *Biochem J* 89: 114, 1963
33. Kendall KG, Stuart A: Inference and relationship. In: *The Advanced Theory of Statistics*, 3rd Ed., London, Griffin, 1973, pp 323–324
34. Hellman RM, Jackson DV, Buss DH: Thrombotic thrombocytopenic purpura and hemolytic-uremic syndrome in HLA-identical siblings. *Ann Intern Med* 93: 283–284, 1980
35. Elias M, Horowitz J, Tal I, Kohn D, Flatau E: Thrombotic thrombocytopenic purpura and hemolytic uremic syndrome in three siblings. *Arch Dis Child* 63: 644–646, 1988
36. McCoy RC, Abramowsky CR, Krueger R: The hemolytic uremic syndrome, with positive immunofluorescence studies. *J Pediatr* 85: 170–174, 1974
37. Hammar SP, Bloomer HA, McCloskey D: Adult hemolytic uremic syndrome with renal arteriolar deposition of IgM and C3. *Am J Clin Pathol* 70: 434–439, 1978
38. Bokisch VA, Müller-Eberhard HJ, Cochrane CG: Isolation of a fragment (C3a) of the third component of human complement containing anaphylatoxin and chemotactic activity and description of an anaphylatoxin inactivator of human serum. *J Exp Med* 129: 1109–1130, 1969

39. Winckistein JA, Sullivan KE, Colten HR: Biologic consequences of complement activation. In: *The Metabolic and Molecular Bases of Inherited Disease*, edited by Scriver CR, Beaudet AL, Sly WS, Valle D, 7th Ed., New York, McGraw-Hill, 1995, pp 3911–3941
40. Nicol PAE, Lachmann PJ: The alternate pathway of complement activation: The role of C3 and its inactivator (KAF). *Immunology* 24: 259–275, 1973
41. Williams DG, Peters DK, Fallows J, Petrie A, Kourilsky O, Morel-Maroger L, Cameron JS: Studies of serum complement in the hypocomplementaemic nephritides. *Clin Exp Immunol* 18: 391–405, 1974
42. Poskitt TR, Fortwengler HP Jr, Lunskis BJ: Activation of the alternate complement pathway by autologous red cell stroma. *J Exp Med* 138: 715–722, 1973
43. Thompson RA, Lachmann PJ: A second case of human C3b inhibitor (KAF) deficiency. *Clin Exp Immunol* 27: 23–29, 1977
44. West CD: Nephritic factors predispose to chronic glomerulonephritis. *Am J Kidney Dis* 24: 956–963, 1994
45. Rey-Campos J, Rubinstain P, De Cordoba SR: A physical map of the human regulator of complement activation gene cluster linking the complement genes CR1, CR2, DAF and C4PB. *J Exp Med* 167: 664–669, 1988
46. Tomar RH, Kolchins D: Complement and coagulation: Serum beta-lc-beta-la in disseminated intravascular coagulation. *Thromb Diath Haemorrh* 27: 389–395, 1972
47. Kalowski S, Howes EL Jr, Margaretten W, McKay DG: Effects of intravascular clotting on the activation of the complement system: The role of the platelet. *Am J Pathol* 78: 525–536, 1975

CHAPTER 3

The molecular basis of familial hemolytic uremic syndrome: mutation analysis of factor H gene reveal a hot spot in short consensus repeat 20

J. Caprioli , P. Bettinaglio, P.F. Zipfel, B. Amadei, E. Daina, S. Gamba, C. Skerka, N. Marziliano, G. Remuzzi and M. Noris, published in

Journal of the American Society of Nephrology 2001;12:297-307

J Am Soc Nephrol 12: 297–307, 2001

The Molecular Basis of Familial Hemolytic Uremic Syndrome: Mutation Analysis of Factor H Gene Reveals a Hot Spot in Short Consensus Repeat 20

JESSICA CAPRIOLI,* PAOLA BETTINAGLIO,* PETER F. ZIPFEL,† BARBARA AMADEI,* ERICA DAINA,* SARA GAMBA,* CHRISTINE SKERKA,† NICOLA MARZILIANO,‡ GIUSEPPE REMUZZI,*§ and MARINA NORIS* on behalf of the Italian Registry of Familial and Recurrent HUS/TTP

**Mario Negri Institute for Pharmacological Research, Clinical Research Center for Rare Diseases, Aldo e Cele Daccò, Villa Camozzi–Ranica, Italy; †Hans-Knöll-Institute for Natural Products Research, Jena, Germany; ‡PE Applied Biosystems, Monza, Italy; and §Unit of Nephrology and Dialysis, Azienda Ospedaliera, Ospedali Riuniti di Bergamo, Italy.*

Abstract. The aim of the present study was to clarify whether factor H mutations were involved in genetic predisposition to hemolytic uremic syndrome, by performing linkage and mutation studies in a large number of patients from those referred to the Italian Registry for Recurrent and Familial HUS/TTP. PCR and Western blot analyses were conducted to characterize the biochemical consequences of the mutations. Five mutations in the factor H gene were identified. Three, identified in two families and in a sporadic case, are heterozygous point mutations within the most C-terminal short consensus repeat 20 (SCR20) of factor H, resulting in single amino acid substitutions. The other two mutations introduce premature stop codons that interrupt the translation of factor H. A heterozygous nonsense mutation was identified in SCR8 in one family, and a homozygous 24-bp deletion within SCR20 was identified in a Bedouin family with a recessive mode of inheritance. Reverse transcription-PCR analysis of cDNA from peripheral blood leukocytes from the Bedouin family showed that the deletion lowered factor H mRNA levels. Although heterozygous mutations were associated with normal factor H levels and incomplete penetrance of the disease, the homozygous mutation in the Bedouin family resulted in severe reduction of factor H levels accompanied by very early disease onset. These data provide compelling molecular evidence that genetically determined deficiencies in factor H are involved in both autosomal-dominant and autosomal-recessive hemolytic uremic syndrome and identify SCR20 as a hot spot for mutations in the disease. The mutations identified here give an important hint to the relevance of the C-terminus of factor H in the control of the alternative complement activation pathway.

Hemolytic uremic syndrome (HUS) is a disease of nonimmune hemolytic anemia, thrombocytopenia, and renal failure caused by platelet thrombi in the microcirculation of the kidney and other organs (1–3). In its typical presentation, HUS manifests as an acute disease and 80 to 90% of cases recover without sequelae, either spontaneously (as in most cases of childhood HUS) or after plasma infusion or exchange (as in adult or severe forms of HUS) (1,2,4). Typical HUS is triggered by environmental factors, drugs, or infective agents such as the shiga-like toxin-producing *Escherichia coli*; systemic immune disorders or cancer may also cause the disease. These forms of HUS may subside when the underlying condition has been treated or removed. However, there are rare forms, often occurring in families, that frequently relapse even after complete recovery of the presenting episode (1,5), with death or permanent neurologic or renal sequelae being the final outcome in the majority of cases. These "atypical" forms are believed to have a genetic background that predisposes to microvascular thrombosis (6,7). Both autosomal-recessive and autosomal-dominant modes of inheritance have been recognized (5,6,8–11), with precipitating events such as pregnancy, virus-like disease, or sepsis being identified in some (12) but not all series (13,14). Evidence that some of these cases could be cured, at least transiently, with plasma infusion or exchange suggested that the underlying genetic defect(s) associated with familial HUS were the cause of one or more abnormalities in plasma component(s) essential to the integrity of the microvascular circulation and/or to the defense mechanism of the host endothelium against injurious agents. In this context, reduced serum levels of the third component (C3) of the complement system had been reported since 1974 in sporadic or familial forms of HUS (15–21). This initially was attributed to an inherited defect in C3 synthesis (22), but more convincing data are now available

Received July 5, 2000. Accepted August 28, 2000.
Dr. Clifford Kashtan served as guest editor and supervised the review and final disposition of this manuscript.
Correspondence to Dr. Jessica Caprioli, Clinical Research Center for Rare Diseases, Aldo e Cele Daccò, Mario Negri Institute for Pharmacological Research, Via Gavazzeni, 11, 24125 Bergamo, Italy. Phone: 390 35 319888; Fax: 390 35 319331; E-mail: caprioli@marionegri.it

1046-6673/1202-0297
Journal of the American Society of Nephrology

that low C3 in HUS derives from either a lack (21,23,24) or altered function (25) of factor H, a regulatory component of the alternative pathway of the complement system (26–28).

In a recent study (12) in a consistent number of families, we provided epidemiologic evidence of an association between factor H deficiency and low C3 levels in familial forms of HUS. Another report (25) documented that an area on chromosome 1q, where factor H is mapped, segregates with HUS. In one family, a mutation in factor H gene was described, consisting of a C to G transversion causing an arginine to glycine change in short consensus repeat 20 (SCR20) (25).

The present study was designed to characterize the genetic defect underlying atypical HUS by genotyping a large number of patients from our Registry for Familial and Recurrent HUS/TTP. Here we describe five mutations; three were found in families with dominant inheritance, one was found in a family with recessive inheritance, and one was found in an adult case of sporadic HUS whose disease recurred in the transplanted kidney. These results provide molecular evidence that the primary abnormality in atypical HUS is overactivity of the alternative complement pathway as a result of impaired factor H function.

Materials and Methods

Patients

HUS was diagnosed in all cases reported to have one or more episodes of microangiopathic hemolytic anemia and thrombocytopenia, defined on the basis of hematocrit <30%, hemoglobin <10 mg/dl, serum lactate dehydrogenase >460 units/L, undetectable serum haptoglobin, fragmented erythrocytes in the peripheral blood smear, and platelet count <150,000/μl, associated with acute renal failure.

HUS was defined as familial when at least two members of the same family were affected by the disease at least 6 mo apart and exposure to a common environmental triggering agent (in particular a shiga-like toxin-producing strain of *E. coli*) could be reasonably excluded.

Plasma levels of the third complement component below the lower limit of normal ranges (calculated as mean ± SD), *i.e.*, <83 mg/dl, were taken as indicating hypocomplementemia.

Microsatellite Polymorphism Genotyping and Linkage Analysis

Genomic DNA was extracted from whole blood according to standard protocols (Nucleon BACC2 kit, Amersham, UK).

Microsatellite polymorphisms flanking and within the gene for complement factor H (HF, chromosome 1q32) were studied in a candidate gene approach to linkage analysis. Markers analyzed in this study are D1S240, D1S202, D1S412, D1S2816, D1S413, D1S2738, and D1S2796; primers were synthesized by Life Technologies (Paisley, UK).

PCR reactions were done in a 20-μl volume containing 100 ng DNA, 17 pmol of each primer, 16 nmol dNTP, 1.5 mM $MgCl_2$, 1 U *Taq* polymerase (*Taq* Gold, PE Applied Biosystems, Foster City, CA), in the presence of ^{32}PdCTP, and PCR buffer. After an initial 10-min denaturation at 94°C, 35 cycles were performed (94°C for 45 s, 54°C for 30 s, and 72°C for 45 s), followed by a final 10-min extension at 72°C. Samples were mixed with 20 μl of loading buffer, denatured at 75°C for 5 min, and electrophoresed on a denaturing 6% (19:1 acryl:bis) acrylamide gel in Tris Borate EDTA buffer, at 55 W for 2 to 4 h. Gels were then exposed to x-ray films for 3 h to overnight.

Linkage analysis was performed using a FASTLINK package for two-point analysis and Genehunter package for multipoint analysis (www.linkage.rockefeller.edu). Autosomal-recessive and autosomal-dominant transmission, with incomplete penetrance (0.7), were taken into consideration; the disease gene frequency in the general population was assumed to be 0.0001.

Single-Strand Conformation Polymorphism Analysis and Sequencing of Factor H

Factor H exons were amplified using primers located in the flanking introns and analyzed by single-strand conformation polymorphism (SSCP) of DNA; primers were constructed to avoid coamplification of highly homologous SCR in factor H related genes (hFHR) (26). For amplification of SCR20, it was necessary to divide the exon into two parts (called SCR20A and SCR20B) that were independently amplified using two couples of primers, constructed to obtain 3′ mismatches on the hFHR-1 gene sequence (see Figure 1). Primers were synthesized by Life Technologies.

A total of 100 ng of genomic DNA were amplified as described above. PCR products were electrophoresed on nondenaturing 6% (62:1 acryl:bis) acrylamide gel in TAE buffer (pH 6.8) at 35 W for 3 to 5 h and at 4 W overnight. Gels were visualized by silver staining,

Figure 1. Alignment between short consensus repeat 20 (SCR20) genomic sequences of factor H and factor H-related 1 (FHR-1). Vertical bars with arrows indicate beginning and ending of SCR20. All mismatched nucleotides are boxed in gray. SCR20 was divided into two segments that were independently amplified using two sets of primers (gray arrows for SCR20B and black arrows for SCR20A).

and aberrant bands were sequenced using an ABI 377 sequencer (PE Applied Biosystems).

mRNA Extraction, cDNA Synthesis, and Analysis

mRNA was extracted from peripheral blood mononuclear cells (PBMC) isolated from whole blood (standard protocol); reverse transcription (RT) was conducted using the Life Technologies Brl protocol with oligo (dT) primers, in a total volume of 20 μl, and the cDNA obtained was used to investigate mRNA levels in family 29. RT-PCR products of SCR20A from heterozygous individuals of family 29 were electrophoresed on a nondenaturing 8% (37.5:1 acryl:bis) acrylamide gel in TBE buffer at 80 W for 2 to 4 h. Gels were then stained with ethidium bromide.

mRNA levels from three heterozygous individuals from family 29 and from healthy control subjects ($n = 3$) were also evaluated by real-time PCR quantification (29) on the ABI Prism 7700 platform (PE Applied Biosystems). Five μl of cDNA were amplified with 2.5 μl of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) PDAR Kit (including forward and reverse primers as well as a specific VIC labeled TaqMan probe, PE Applied Biosystems), and 25 μl of TaqMan Universal MasterMix (PE Applied Biosystems) in a final volume of 50 μl. The amplification profile consisted of 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s, then 60°C for 1 min. In parallel 5 μl cDNA were amplified using 25 μl of SYBR Green I (30), TaqMan Universal Master Mix (PE Applied Biosystems), primers for SCR20A, in a final volume of 50 μl with an amplification profile of 2 min at 50°C, 10 min at 95°C and then 40 cycles of 15 s at 95°C, 20 s at 55°C, and 30 s at 72°C. The amplification curve was obtained using fluorescence values collected at 72°C.

After GAPDH normalization, mRNA levels of SCR20A (target gene) in heterozygous individuals were quantified as a percentage of mRNA levels in healthy control subjects. We applied the ΔΔCycles threshold (ΔΔCt) method (User bulletin #2, PE Applied Biosystems and references 31–33). Melting temperature analysis (30) was also performed: at the end of amplification reaction, samples were denatured at 95°C and then slowly cooled to 60°C in 20 min. The amplification product was verified by acrylamide gel. No primer-dimers or aspecific amplification products were evidenced by melting temperature analysis and acrylamide gel.

Factor H and C3 and C4 Quantification

Factor H was quantified by radial immunodiffusion assay using a sheep polyclonal anti-human factor H antiserum, which reacts with various epitopes of factor H molecule (The Binding Site, Birmingham, UK). C3 and C4 were quantified by kinetic nephelometric measurements.

Western Blot Analysis of Factor H

To search for possible qualitative and quantitative defects of the different factor H molecules, Western blot analysis of factor H, factor H-like and factor H-related proteins in serum samples was also performed. Serum (1.5 μl) was separated by 10 or 7% sodium dodecyl sulfate polyacrylamide gel electrophoresis according to Laemmli using prestained bench markers (Life Technologies) as standards. Proteins were electroblotted to nitrocellulose by semidry blotting. Membranes were blocked for 30 min using 5% (wt/vol) dried milk in phosphate-buffered saline (PBS). Incubations with polyclonal goat anti-factor H antiserum (Calbiochem, San Diego, CA; diluted 1:1000) or anti FHL-1 rabbit anti-SCR1 to 4 antiserum, that does not detect FHR-1 and FHR-2 proteins (dilution 1:1000 (34)) were performed at 4°C overnight. After the membranes were washed five times in PBS, they were incubated with peroxidase-conjugated rabbit anti-goat or swine anti-rabbit antibody (Dako, Hamburg, Germany) for 2 to 3 h. Protein bands were visualized by the addition of 0.3% (wt/vol) 4-chloro-1-naphthol in 10% (vol/vol) methanol in PBS.

Results

Patients

Family Pedigrees and Sporadic Case Report. From the database of the Italian Registry for Recurrent and Familial HUS/TTP (12), four families (1, 3, 24, and 29) were selected for the present study on the basis of persistent hypocomplementemia in all cases. A female with sporadic HUS (R16) who experienced disease recurrence in the renal allograft was also studied.

As reported in Figure 2, in families 1, 24, and 29, the onset of the disease was during infancy (from a few weeks to 8 yr), whereas in family 3, the disease became manifest in adulthood.

Conditions that potentially predispose to the disease were recognized in all cases from families 1 and 24 (upper respiratory tract infection) and in patient F39#3 (pregnancy). One or more disease relapses were reported in all cases but one (F40#3). Eleven participants had died before the study was conducted. Among the seven survivors, four are on chronic dialysis; of these, two (F34#1 and F39#3) received a kidney transplant that failed because of recurrence of disease.

The pedigrees of the four families and haplotypes on 1q32 are shown in Figure 2. C3 concentrations were below the normal range in all patients and in some of their relatives (Table 1), whereas C4 concentrations were normal, indicating selective activation of the alternative complement pathway.

The pattern of inheritance of HUS could not be established in family 1, because only one patient was alive at the time of the study. No consanguinity was inferred. The pedigree was scarcely informative, with either the dominant or the recessive hypothesis (Table 2). In families 3 and 24, no consanguinity was reported; either recessive or dominant mode of inheritance could fit the haplotype data (Figure 2) and linkage results (Table 2). In family 29, a recessive inheritance was hypothesized because of the high grade of consanguinity; this was confirmed by the haplotype data (Figure 2) and logarithm of odds score values (Table 2). Results of SLINK simulation are reported in Table 2 as an index of the informativeness of the pedigrees.

Sporadic Case. In 1997, a 48-yr-old woman, who had been on chronic hemodialysis since 1995 because of progressive loss of renal function after an HUS episode in 1980, received in our Transplant Department a cadaveric renal allograft, under cover of steroid plus cyclosporine-based immunosuppression. Graft function deteriorated acutely on day 7 post-transplantation, and allograft biopsy showed recurrent HUS. A partial improvement was achieved with plasma exchange, but this was not sustained and the graft failed 3 mo later. She is currently on chronic peritoneal dialysis. No relevant clinical history was reported in her family.

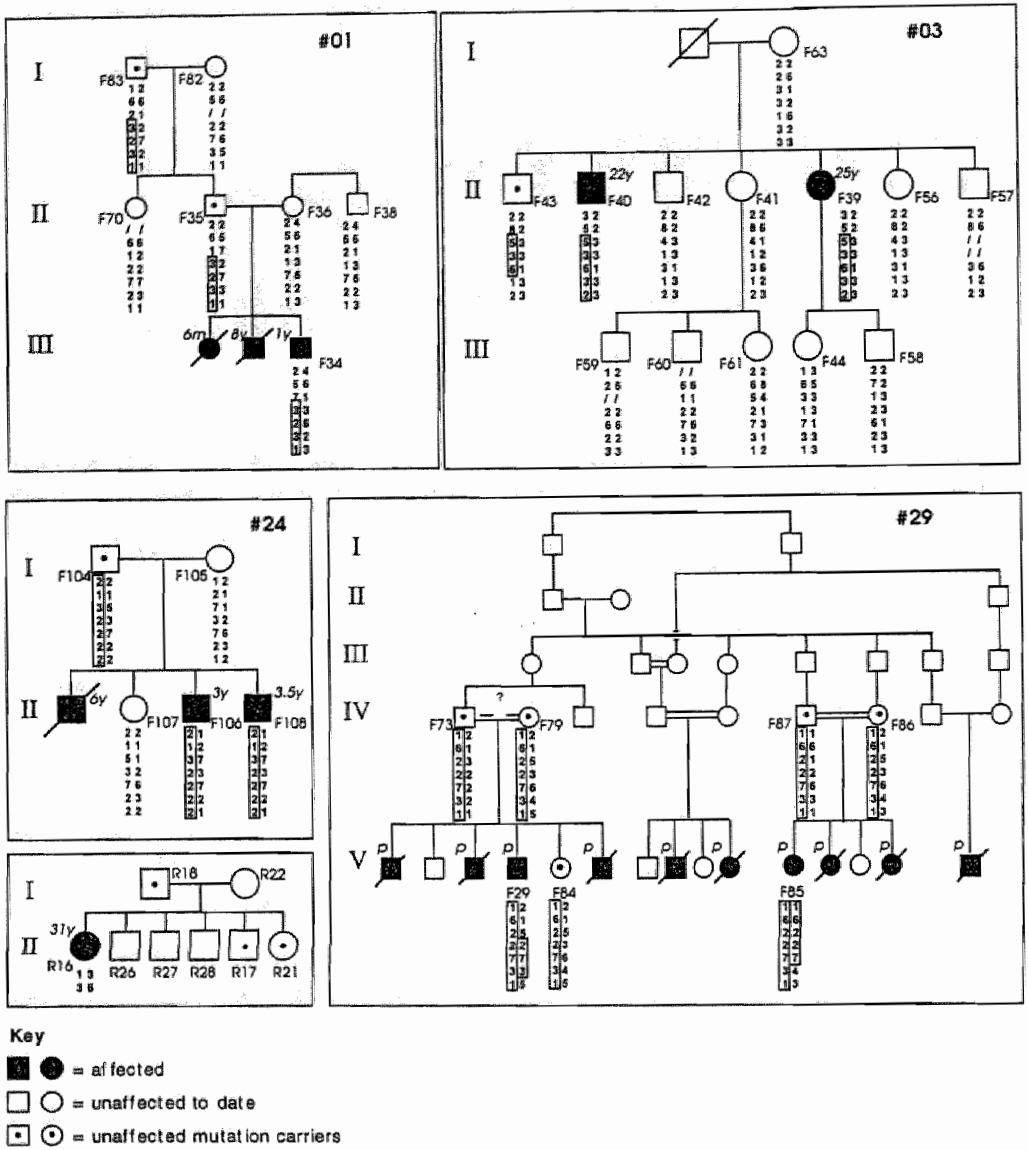


Figure 2. Pedigrees of the four families studied (indicated by #...) and haplotypes on chromosome 1q32 markers (D1S240, D1S202, D1S412, D1S2816, D1S413, D1S2738, D1S2796) flanking factor H gene. The pedigree and the haplotype (markers D1S2816, D1S413) of the sporadic case also are presented. Patients studied are indicated by a number (F... for familial R... for sporadic). Age at onset is indicated in italics; *p*, perinatal onset. Chromosomes carrying mutations are boxed.

SSCP and Sequencing Results

PCR fragments corresponding to the 20 SCR of the factor H gene were analyzed for mutation screening by SSCP followed by sequencing of aberrant bands. SSCP detected a band shift in all cases and carriers from the four families as identified by haplotype analysis. The aberrant bands were localized in SCR8 in family 3 and in SCR20 in families 1, 24, and 29. The sporadic case showed an aberrant band in SCR20, which was also found in the father and two of five healthy siblings. None of the aberrant bands was found in 100 normal chromosomes.

Table 1. Complement profiles[a]

Family	Subject	C3 (mg/dl) (83–177)*	C4 (mg/dl) (15–45)*	Factor H (mg/L) (350–650)*
1	F83	84	27	476
1	F35	**76**	20	496
1	**F34**	**69**	15	561
1	F36	105	26	550
3	F63	101	19	662
3	F43	92	22	446
3	**F40**	**68**	18	569
3	F42	95	22	679
3	F41	**73**	17	537
3	**F39**	**51**	26	386
3	F44	**79**	**12**	529
3	F58	117	25	–
3	F56	**78**	15	616
3	F57	100	18	523
24	F104	130	332	835
24	F105	97	24	546
24	F107	96	27	562
24	**F106**	**61**	16	711
24	**F108**	**61**	15	569
29	**F29**	**14**	42	**<70**
29	F73	133	42	344
29	F79	135	49	252
29	F84	107	24	358
29	**F85**	**10**	—	**<70**
29	F86	103	34	362
29	F87	86	36	274
	R16	122	28	731

[a] Complement profiles in the four families studied and in the sporadic case. Affected subjects and low values of C3, C4, and factor H are indicated in bold.
*, normal ranges.

Mutation Description and Biochemical Characterization of Factor H

Family 1. Sequence analysis revealed a heterozygous 3717 G to A transition; this results in an amino acid change of arginine to glutamine (R1215Q). This mutation was present in the patient and his unaffected father and his unaffected grandfather (Figure 3, panel A). Factor H protein levels (Table 1) and the Western blot profile were normal both in the case and his relatives (Figure 4A).

Family 3. A heterozygous bp deletion was found in the two patients and one unaffected brother involving one of the three adenines from position 1494 to 1496; this mutation causes a frame shift that results in the formation of a short sequence of five anomalous codons and a premature stop codon that interrupts the transcription within SCR8 (Figure 3, panel B). The same mutation was evident also on the RT-PCR product of RNA extracted from PBMC, indicating that the mutated DNA is transcribed (Figure 3, panel B1).

Radial immunodiffusion assay found that factor H levels in carriers of the mutation were in the normal range (Table 1). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis (Figure 4B) of sera in this family showed that the 150-kD factor H band was reduced in the mutation carriers. The truncated protein was not identified in these patients by Western blotting; however, the truncated protein could have been masked by factor H-like 1 (26), which derives from an alternative splicing of factor H primary transcript, as a result of the very close molecular weight of the two proteins.

Family 24. A heterozygous 3701 C to T transition that determines an arginine to cysteine change (R1210C) was found (Figure 3, panel C). This mutation is present in the two patients and their unaffected father. Thus, the patients seem to have inherited the abnormal factor H allele from their father. Although factor H protein levels in the patients' plasma were normal, Western blot experiments revealed additional high-molecular-weight bands that reacted with anti-factor H antiserum in the two affected patients and their healthy father. These bands were absent in their healthy mother and sister and in control subjects (Figure 4, C and D). The nature of these bands with lower mobility is not clear. One possible explanation could be the formation of factor H dimers caused by intermolecular disulphide bonding with the additional cysteine in SCR20.

Family 29. An A to T transversion and a 24-bp deletion was found in homozygosity in affected members of family 29 (Figure 3, panel E). This mutation was also present in heterozygosity (Figure 3, panel D) in all of the other family members, as supposed by haplotype analysis (Figure 2). The deletion causes a stop codon within SCR20 and the loss of the four most C-terminal amino acids.

Ying *et al.* (35) described an apparent exchange at position 3645 in SCR20 in this Bedouin family. However, they used a set of primers that are complementary to both HF and FHR-1. While amplifying DNA from three healthy control subjects using this set of primers, we found the same base exchange as Ying *et al.* (35) and a further nucleotide mismatch that exactly corresponds to FHR-1.

The factor H concentration was abnormally low in this family: factor H serum levels were severely depressed in the two affected patients (Table 1 and reference 12) and below the normal range in the heterozygous subjects. The Western blot patterns of factor H in this family confirmed the low protein levels in the two homozygous patients, although some residual protein was detectable (12).

We used PBMC to conduct RT-PCR analysis on SCR20 to determine how this deletion affected mRNA levels. Upon separation of the cDNA PCR products from heterozygous carriers by acrylamide gel electrophoresis, we found two bands of different intensities: a 178-bp band corresponding to the wild-type cDNA—as evidenced by cDNA analysis from normal control subjects—and a second 154-bp band corresponding to the deleted cDNA (Figure 5A). The intensity of the aberrant band was much lower than the wild-type band (Figure 5A), suggesting a selective defect in transcription or stability of

Table 2. LOD scores obtained by two-point analysis between hemolytic uremic syndrome and seven markers around factor H[a]

	Dominant Hypothesis (θ = 0,0)[b]					Recessive Hypothesis (θ = 0,0)				
	#1	#3	#24	#29		#1	#3	#3[a]	−24	−29
D1S240	−0.14	0.88	0.31	0.03	D1S240	0.00	0.93	0.72	0.43	0.21
D1S202	0.01	0.88	0.31	−0.23	D1S202	0.00	1.23	1.02	0.43	0.44
D1S412	−0.16	0.68	0.49	0.30	D1S412	0.00	−1000	0.41	0.73	−1000
D1S2816	−0.15	0.34	0.00	0.22	D1S2816	0.00	−1000	0.41	0.12	0.69
D1S413	0.20	0.48	0.49	0.26	D1S413	0.00	−1000	0.50	0.73	1.58
D1S2738	0.04	1.03	0.31	−0.19	D1S2738	0.00	1.23	1.02	0.43	−3.97
D1S2796	−0.21	0.10	0.31	−0.28	D1S2796	0.00	−0.01	−0.00	0.43	−1000
SLINK[b]	0.31	1.40	0.49	1.07	SLINK[b]	0.00	1.23	1.02	0.73	2.10

[a] LOD, logarithm of odds.
[b] Penetrance 0.7.
[c] Maximum value obtained by SLINK simulation.

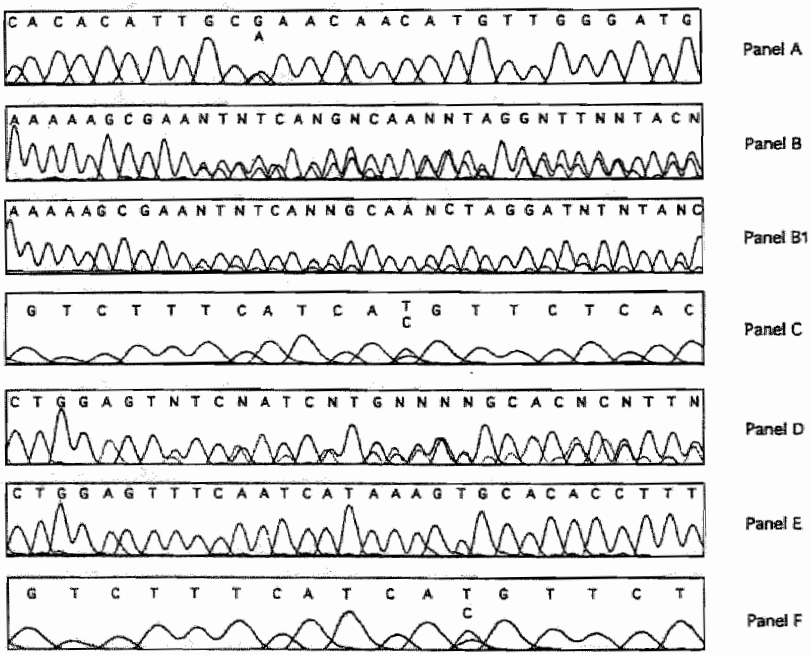


Figure 3. Partial chromatograms of SCR8 (panels B and B1) and 20 (panels A and C to F) sequences describing the mutations in our series. (Panel A) DNA from subject F34#1 (familial hemolytic uremic syndrome [HUS]), heterozygous G to A transition. (Panel B) DNA from subject F39#3 (familial HUS), heterozygous frame shift caused by an A deletion. (Panel B1) cDNA from the same patient: the heterozygous A deletion is present also in the cDNA. (Panel C) DNA from patient F106#24 (familial HUS), heterozygous C to T transition. (Panel D) DNA from patient F86#29 (healthy carrier), heterozygous A to T transversion and frame shift caused by a 24-bp deletion. (Panel E) DNA from patient F29#29 (familial HUS), homozygous A to T transversion and frame shift caused by a 24-bp deletion. (Panel F) DNA from patient R16 (sporadic HUS), heterozygous C to T transition.

the mutated mRNA. Thus, quantitative real-time PCR showed approximately a 50% reduction of mRNA levels in heterozygous individuals (Figure 5B). Melting temperature analysis showed two peaks in heterozygous subjects corresponding to the wild-type and the deleted cDNA. Unfortunately, RNA from homozygous patients was not available.

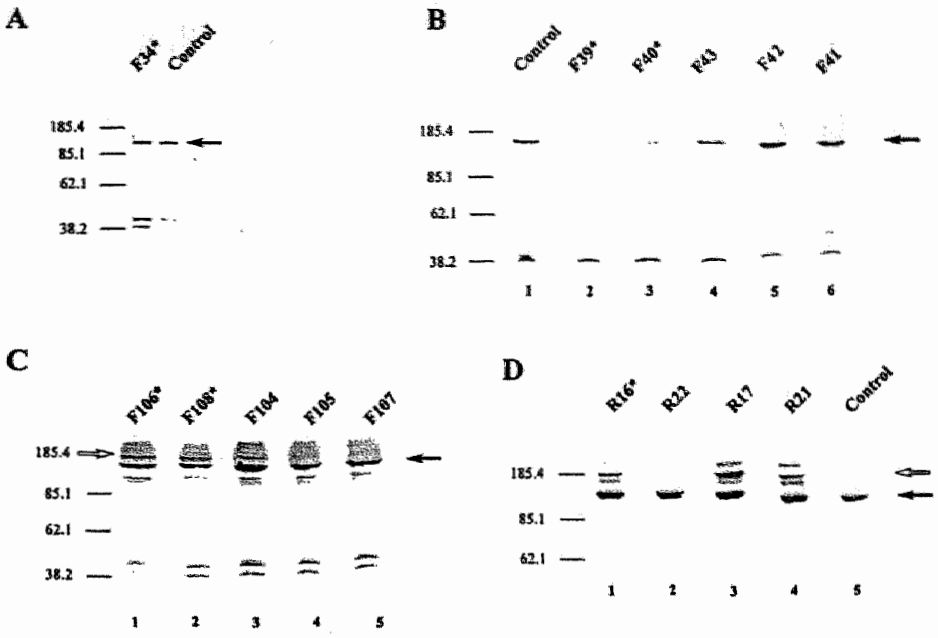


Figure 4. Western blot analysis of factor H in human serum. Sera were separated by 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the Western blots were developed with polyclonal rabbit anti-factor H antiserum, specific for factor H and FHL-1/reconectin. Closed arrows indicate factor H, and open arrows point to the abnormal high-molecular-weight bands in C and D. Patients are marked by an asterisk. The mobility of the size markers (kD) is indicated. (A) Family 1, sera from the patient still alive (F34) and from a healthy control subject are shown. (B) Family 3, sera from the two patients (F39 and F40), the carrier brother (F43), and two healthy relatives (F42 and F41) are shown. (C) Family 24, sera from the patients still alive (F106 and F108), the carrier father (F104), the healthy mother (F105), and the healthy sister (F107) are shown. (D) Sera from the patient with sporadic HUS (R16), the healthy mother (R22), the two carrier healthy siblings (R17 and R21), and a healthy control subject are shown.

Sporadic Case. A heterozygous mutation was found in the patient who experienced a sporadic episode of HUS. The mutation, a 3701 C to T transition, results in the change of an arginine to cysteine (R1210C) and is identical to that found in family 24, (Figure 3, panel F). We were unable to find any familial relationship between the sporadic patient, who is from the north of Italy, and family 24, which is from the south. Analysis of markers D1S2816 and D1S413 showed that this patient does not share the haplotypes of family 24 (Figure 2); thus, the possibility of a common foundation event is unlikely.

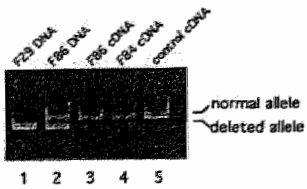
The mutation was also found in the father and two of five healthy siblings. Serum factor H levels were within the normal range (Table 1). Western blot experiments revealed in the sporadic case the presence of the same additional high-molecular-weight bands as in family 24. These bands were also present in all relatives who carried the mutation (Figure 4D).

Discussion

In the present study, we identified five mutations in the factor H gene in four families and one sporadic case of HUS. Three are point mutations within the most C-terminus SCR of factor H (SCR20), resulting in single amino acid substitutions. Interestingly, an R1210C mutation was identified separately in one family and in one unrelated individual who developed a sporadic form of the disease. In another family, an R1215Q substitution was identified. A mutation in the same codon has been previously reported by Warwicker *et al.* (25) in a family with HUS, which resulted, however, in a different amino acid exchange (R1215G).

We also identified two mutations that lead to the formation of truncated factor H proteins. Specifically, a nonsense mutation was found in one family, which introduces a premature stop codon. This mutation results in a truncated protein that consists of SCR1 to 8 and has the last twelve SCR deleted. A 24-bp deletion within SCR20 was found in a Bedouin family, resulting in a protein having the most C-terminal end truncated. The 24-bp deletion was present in homozygosity in patients and in heterozygosity in their relatives, consistent with a recessive mode of inheritance. This pattern distinguishes this family from all of the others, in which all affected members were heterozygous for factor H mutations, consistent with a dominant pattern. Two groups have already studied the Bedouin family for factor H mutations. Ying *et al.* (35) described an apparent exchange of a single nucleotide at position 3645 in

A



B

subject	ΔCt	ΔΔCt	mRNA*	T melting	
F73	12.3±0.02	1	50%	77.9±0.03	81.9±0.02
F84	12.4±0.03	1.1	47%	77.8±0.01	81.6±0.03
F86	12.2±0.04	0.9	54%	77.5±0.04	81.5±0.04
controls+	11.3±0.01				81.8±0.03

Figure 5. Quantification of mRNA from patients of family 29. (A) Electrophoresis of PCR products: lane 1, DNA from F29 (homozygous for the 24-bp deletion); lane 2, DNA from F86 (heterozygous for the 24-bp deletion); lanes 3 and 4, cDNA from F86 and F84 (heterozygous for the 24-bp deletion); lane 5, cDNA from a healthy control subject. Levels of the deleted mRNA are clearly low in the heterozygous patients. (B) Real-time PCR results: *mRNA levels are expressed as percentage of levels in healthy control subjects ($n = 3$) after normalization for the housekeeping gene GAPDH. Each sample was run in triplicate; results are expressed as mean ± SD of the three runs. +, mean of three controls.

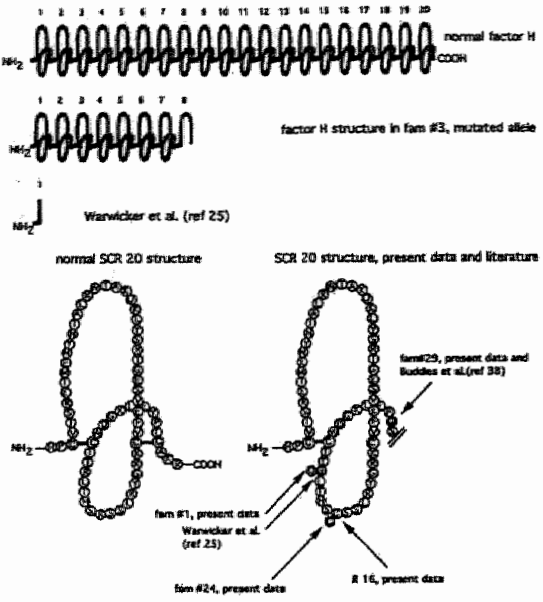


Figure 6. Summary of all seven mutations reported in the factor H gene of HUS patients. Three mutations introduce premature stop codons within SCR1, SCR8, and SCR20; the other four mutations affect arginine residues (R1210 and R1215). With the exception of the deletion within SCR20 (Bedouin family), all mutations are heterozygous.

SCR20, and Buddles *et al.* (36) reported the 24-bp deletion that we confirm here. Data reported by Ying are very likely artifacts due to co-amplification of the strongly homologous FHR-1 gene (37), as the forward and reverse primers that they used are complementary to both factor H and FHR-1. This is confirmed by our findings in healthy control subjects that the same primers amplified a DNA fragment with the same base exchange described by Ying *et al.* and a further nucleotide mismatch that exactly corresponds to FHR-1.

Thus, the new mutations presented here, together with the previously published data (25,36) (Figure 6), provide strong molecular evidence that factor H is involved in both the dominant and the recessive forms of HUS. None of the mutations were ever found in healthy control subjects, excluding the possibility that they are common polymorphisms in the normal population. The point mutations (reference 25 and present data) all were inherited in an autosomal-dominant mode. By contrast, nonsense mutations gave rise to heterogeneous phenotypes; thus, a stop codon in SCR8 was associated with a dominant pattern of inheritance and late onset of the disease, whereas a stop codon in SCR20 produced a recessive pattern with a very early onset. Finally, a third nonsense mutation, located in SCR1, has already been found (25) in a sporadic case of HUS with late onset.

What seems to distinguish dominant and recessive forms are the biochemical and clinical consequences of the mutations: dominant inheritance is associated with normal factor H levels and an abnormal protein in the circulation. In these cases, an incomplete penetrance was observed; indeed, approximately 40% of carriers did not develop HUS. In most cases, the disease developed during late infancy or adulthood, often as a consequence of events such as infection or pregnancy, which are known to be triggering factors for HUS (1,2). This suggests that in dominant forms, haploinsufficiency of factor H, which is compensated by the normal allele in basal conditions, requires intercurrent environmental or acquired factors to manifest fully. By contrast, the mutation that causes autosomal-recessive HUS in the Bedouin family resulted in a severe reduction of serum factor H levels, accompanied by very early onset of the disease (1 to 20 wk) and no recognized trigger. Factor H reduction in this family may be due either to impaired secretion of the mutant protein, consequent to the loss of a disulphide bridge essential for protein folding and secretion (38), or to reduced mRNA levels as a result of premature arrest of translation (39). The latter possibility is supported by our results of mRNA analysis in PBMC from heterozygous carriers, in which the expression of the mutated allele was lower than the normal allele.

A number of reports have claimed an association between factor H deficiency and recurrent (22), atypical (24), and familial (12) forms of HUS. However, factor H deficiency has also been associated with an increased tendency to other dis-

eases, including systemic lupus erythematosus (40), pyogenic infection susceptibility (41), and a patient with membranoproliferative glomerulonephritis with collagen III deposition in the mesangium (42). In the latter patient, Ault *et al.* (43) identified two point mutations in SCR9 and SCR16, which cause abnormal secretion and severe factor H deficiency.

Why different mutations in the same gene can cause different diseases cannot be fully clarified on the basis of the present and previous data, and further investigations on the genotype–phenotype correlations are required. However, the finding that five of the seven mutations described until now in HUS are located in SCR20 of complement factor H identifies this domain as a mutational hot spot for HUS. It also suggests the presence, in the very C-terminus of factor H, of highly relevant functional sites whose loss is involved in the pathogenesis of this disease.

Factor H binds C3b and controls the alternative pathway of complement activation in two ways. First, it accelerates the decay of the alternative pathway C3 convertase (C3bBb) by displacing factor B. Second, it acts as a cofactor for factor I–mediated inactivation of C3b (cofactor activity) (26).

Factor H has at least three C3b binding domains, one of which resides within SCR 16 to 20 (44). In addition, SCR7 and 19 to 20 contain two major binding sites for heparin and heparin-like proteins, such as sialic acid on glycoproteins and glycolipids (45). All of these functions are very likely lost in mutated factor H in family 3, in which the entire 9 to 20 SCR segment was deleted. The three point mutations that cause arginine substitutions in SCR20, described here, might impair the heparin-binding capacity of factor H as this involves lysine and arginine residues (46,47).

Interaction of factor H with sialic acids and other polyanions on human cells and tissues increases the affinity of factor H for C3b and enhances its inhibitory effect on the alternative pathway of complement activation (48). This control step protects host cells from autolytic attack of the alternative complement pathway, because complement activation induces the deposition of C3b indiscriminately onto host and foreign particles. This applies particularly to endothelial cells, with their high surface density of heparin-like glycosaminoglycans (48). Thus, when an environmental trigger activates the alternative complement pathway, modification of the heparin-binding capacity of mutated factor H might facilitate the occurrence of microvascular endothelial damage.

The present data represent a step forward in understanding the pathogenesis of the microangiopathic process in HUS. We propose that intercurrent exposure to agents that damage the vascular endothelium, such as certain viruses, bacteria, toxins, immunocomplexes and cytotoxic drugs (1,2), may initiate local unrestricted complement activation within capillary vessels (49,50). In normal conditions, however, by modulating C3bBb activity (26–28), factor H may efficiently limit complement deposition, thus preventing any further enhancement of the activation. On the contrary, when the bioavailability or the activity of factor H is congenitally defective, C3bBb convertase formation and complement activation become uncontrolled and result in microangiopathic damage, which leads to full manifestation of the disease.

No specific therapy is effective in familial cases of HUS. Infusion of fresh-frozen plasma, or plasmapheresis, which is effective in recurrent and atypical adult cases, often is inactive in patients with the familial form of the disease (1,5), possibly because of insufficient amounts of factor H in the plasma infused. Replacement with recombinant factor H protein or the use of specific complement inhibitors could represent future perspectives for the treatment of the disease as alternatives to whole plasma.

Note Added in Proof

After the manuscript of this article was submitted for publication, we analyzed a sporadic patient, with low levels of C3 and factor H and with very early onset of HUS (6 mo); we found a mutation in SCR20 of factor H consisting of a heterozygous T3663C transversion that determines V1197A change.

Acknowledgments

We thank Dr. Piero Ruggenenti, Dr. Beatrice Vasile and Dr. Elena Bresin for clinical advice and Dr. Silvia Orisio for her contribution in setting up the methods. Fabio Sangalli helped us for statistical analysis of linkage data. Dr. Judy Baggot edited the manuscript. We deeply are indebted to all participating members of the families and to their referring clinicians: Dr. C. Cascone, Dr. D. Landau, Dr A. Edefonti, Dr. G. Barbano, and Dr. S. Bassi.

We are grateful to the Comitato 30 ore per la vita and to Credito Bergamasco for supporting the study.

Dr. Jessica Caprioli is a recipient of the fellowship in memory of Maria Paola Casirati. Dr. Paola Bettinaglio is a recipient of the fellowship from Associazione Ricerca Malattie Rare (ARMR, Delegazione di Milano) in memory of Prof. Cosimo Conterno. Dr. Barbara Amadei received a fellowship from Associazione Ricerca Malattie Rare (ARMR, Bergamo) through the generosity of Vin Service Srl, Zanica, Bergamo. Dr. Peter F. Zipfel's work is supported by the Deutsche Forschungsgemeinschaft (DFG) Zi 432.

Portions of this work were presented at the XVIIIth International Complement Workshop, July 23–27, 2000, and at the 33rd Annual Meeting of the American Society of Nephrology, October 13–18, 2000.

Appendix

Italian Registry for Recurrent and Familial HUS/TTP

Coordinators. P. Ruggenenti, MD, M. Noris, Chem. Pharm. D., G. Remuzzi, MD (Clinical Research Centre for Rare Diseases *Aldo e Cele Daccò*, Ranica).

Investigators that referred cases to the Registry. F. Casucci, MD, F. Cazzato, MD ("Miulli" Hospital, Acquaviva delle Fonti, Bari); **P. Ponce, MD** (Garcia de Orta Hospital, Bairro do Motadoro, Portugal); **F. Della Grotta, MD** (Polyspecialized Hospital, Anzio, Roma); **R. Bellantuono, MD, T. De Palo, MD** ("Giovanni XXIII" Pediatric Hospital, Bari); **D. Landau, MD** (Soroka Medical Center, Beer-Sheba, Israel); **T. Barbui, MD** (Riuniti Hospitals, Bergamo); **R. Wens, MD** (CHU Brugmann, Bruxelles, Belgium); **J. Ferraris, MD** (Italian Hospital, Buenos Aires, Argentina); **A.**

Cao, MD ("Università degli Studi," Cagliari); **C. Cascone, MD, G. Delfino, MD** ("S. Giacomo" Hospital, Castelfranco Veneto, Treviso); **T. Ring, MD** (Aalborg Hospital, Denmark); **C. Cappelletti, MD, C. Ciccarelli, MD** ("S. Giovanni di Dio" Hospital, Firenze); **G.C. Barbano, MD** ("G. Gaslini" Institute, Genova); **G.B. Haycock, MD** (Guy's Hospital, London, United Kingdom); **A. Bettinelli, MD** ("G. e D. De Marchi" Pediatric Clinic, Milano); **Edefonti, MD** ("G. e D. De Marchi" Pediatric Clinic, Milano); **E. Rossi, MD** ("L. Sacco" Hospital, Milano); **V. Toschi, MD** ("San Carlo Borromeo" Hospital, Milano); **S. Bassi, MD** ("Umberto I" Hospital, Brescia); **D. Belotti, Biol. Sci. D., E. Pogliani, MD** ("San Gerardo" Hospital, Monza, Milano); **A. Indovina, MD, R. Marcenò, MD** ("V. Cervello" Hospital, Palermo); **G. Enia, MD** (Division of Nephrology, Fisiology Clinic Centre, CNR, Reggio Calabria); **F. Mallamaci, MD, C. Zoccali, MD** ("Bianchi-Melacrino-Morelli" Hospital, Reggio Calabria); **A. Amendola, MD, F. Mandelli, MD** ("Università degli Studi La Sapienza," Roma); **A. Gianviti, MD, G.F. Rizzoni, MD** ("Bambino Gesù" Pediatric Hospital, Roma); **T. Cicchetti, MD, G. Putortì, MD** ("N. Giannettasio" Hospital, Rossano Calabro); **A. Pinto, MD** ("San Giovanni di Dio e Ruggi D'Aragona" Hospital, Salerno); **E. Nesti, MD** (S. Miniato Hospital, Firenze); **M. Sanna, MD** (Hospital of Sassari); **A. Amore, MD, R. Coppo, MD** ("Regina Margherita" Pediatric Hospital, Torino); **A. Khaled, MD** ("S. Chiara" Hospital, Trento); **O. Amatruda, MD** ("Fondazione Macchi" Hospital, Varese).

Laboratory Analysis. A. Crippa, MD, A. Vernocchi, MD (Riuniti Hospitals, Bergamo); **F. Gaspari, Chem. D.** (Clinical Research Centre for Rare Diseases *Aldo e Cele Daccò*, Ranica, Bergamo).

References

1. Remuzzi G, Ruggenenti P, Bertani T: Thrombotic microangiopathy. In: *Renal Pathology with Clinical and Functional Correlations*, edited by Tisher CC, Brenner BM, Philadelphia, JB Lippincott Company, 1994, pp 1154–1184
2. Remuzzi G, Ruggenenti P: The hemolytic uremic syndrome. *Kidney Int* 47: 2–19, 1995
3. Kaplan BS, Meyers KE, Schulman SL: The pathogenesis and treatment of hemolytic uremic syndrome. *J Am Soc Nephrol* 9: 1126–1133, 1998
4. Ruggenenti P, Remuzzi G: The pathophysiology and management of thrombotic thrombocytopenic purpura. *Eur J Hematol* 56: 191–207, 1996
5. Kaplan BS, Kaplan P: Hemolytic uremic syndrome in families. In: *Hemolytic Uremic Syndrome and Thrombotic Thrombocytopenic Purpura*, edited by Kaplan BS, Trompeter RS, Moake JL, New York, Marcel Dekker, Inc., 1992, pp 213–225
6. Berns JS, Kaplan BS, Mackow RC, Hefter LG: Inherited hemolytic uremic syndrome in adults. *Am J Kidney Dis* 19: 331–334, 1992
7. Fitzpatrick MM, Dillon MJ, Barratt TM, Trompeter RS: Atypical hemolytic uremic syndrome. In: *Hemolytic Uremic Syndrome and Thrombotic Thrombocytopenic Purpura*, edited by Kaplan BS, Trompeter RS, Moake JL, New York, Marcel Dekker, Inc., 1992, pp 163–178
8. Kirchner KA, Smith RM, Gockerman JP, Luke RG: Hereditary thrombotic thrombocytopenic purpura: Microangiopathic hemolytic anemia, thrombocytopenia, and renal insufficiency occurring in consecutive generations. *Nephron* 30: 28–30, 1982
9. Bergstein J, Michael A Jr, Kjellstrand C, Simmons R, Najarian J: Hemolytic-uremic syndrome in adult sisters. *Transplantation* 17: 487–489, 1974
10. Pirson Y, Lefebvre C, Arnout C, de Strihou CY: Hemolytic uremic syndrome in three adult siblings: A familial study and evolution. *Clin Nephrol* 28: 250–255, 1997
11. Carreras L, Romero R, Requesens C, Oliver AJ, Carrera M, Clavo M, Alsina J: Familial hypocomplementemic hemolytic uremic syndrome with HLA-A3, B7 haplotype. *JAMA* 245: 602–604, 1981
12. Noris M, Ruggenenti P, Perna A, Orisio S, Caprioli J, Skerka C, Vasile B, Zipfel PF, Remuzzi G, on behalf of the Italian Registry of Familial and Recurrent HUS/TTP: Hypocomplementemia discloses genetic predisposition to hemolytic uremic syndrome and thrombotic thrombocytopenic purpura: Role of factor H abnormalities. *J Am Soc Nephrol* 10: 281–293, 1999
13. Feest TG, Hamilton W, Imong S: Severe ocular and pulmonary manifestations of familial hemolytic uremic syndrome: Possible benefit of bilateral nephrectomy. Proceedings of the Twenty-third Congress of EDTA-ERA-Budapest 26, 1986
14. Bukowski RM: Thrombotic thrombocytopenic purpura: A review. In: *Progress in Hemostasis and Thrombosis*, edited by Spaet TH, New York, Grune & Stratton, 1982, pp 287–337
15. Barre P, Kaplan BS, de Chadarevian JP, Drummond KN: Hemolytic uremic syndrome with hypocomplementemia, serum C3NeF, and glomerular deposits of C3. *Arch Pathol Lab Med* 101: 357–361, 1977
16. Monnens L, Molenaar J, Lambert PH, Proesmans W, van Munster P: The complement system in hemolytic-uremic syndrome in childhood. *Clin Nephrol* 13: 168–171, 1980
17. Robson WLM, Leung AKC, Fick GH, McKenna AI: Hypocomplementemia and leukocytosis in diarrhea-associated hemolytic uremic syndrome. *Nephron* 62: 296–299, 1992
18. Kim Y, Miller K, Michael AF: Breakdown products of C3 and factor B in hemolytic-uremic syndrome. *J Lab Clin Med* 89: 845–850, 1977
19. Zachwieja J, Strzykala K, Golda W, Maciejewski J: Familial, recurrent hemolytic-uremic syndrome with hypocomplementemia. *Pediatr Nephrol* 6: 221–222, 1992
20. Landau D, Ohali M, Shalev H, Schlezinger M, Maor E, Carmi R: Immune-mediated pathogenesis of familial hypocomplementemic hemolytic uremic syndrome (HUS) [Abstract]. *J Am Soc Nephrol* 7: 1616, 1996
21. Pichette V, Quérin S, Schürch W, Brun G, Lehner-Netsch G, Delaghe J-M: Familial hemolytic-uremic syndrome and homozygous factor H deficiency. *Am J Kidney Dis* 26: 936–941, 1994
22. Roodhooft AM, McLean RH, Elst E, Van Acker KJ: Recurrent hemolytic uremic syndrome and acquired hypomorphic variant of the third component of complement. *Pediatr Nephrol* 4: 597–599, 1990
23. Thompson RA, Winterborn MH: Hypocomplementemia due to a genetic deficiency of b1H globulin. *Clin Exp Immunol* 46: 110–119, 1981
24. Rougier N, Kazatchkine MD, Rougier J-P, Fremeaux-Bacchi V, Blouin J, Deschenes G, Soto B, Baudouin V, Pautard B, Proesmans W, Weiss E, Weiss L: Human complement factor H deficiency associated with hemolytic uremic syndrome. *J Am Soc Nephrol* 9: 2318–2326, 1998
25. Warwicker P, Goodship THJ, Donne RL, Pirson Y, Nicholls A, Ward RM, Turnpenny P, Goodship JA: Genetic studies into

inherited and sporadic hemolytic uremic syndrome. *Kidney Int* 53: 836–844, 1998
26. Zipfel PF, Jokiranta TS, Hellwage J, Koistinen V, Meri S: The factor H-protein family. *Immunopharmacology* 42: 53–60, 1999
27. Nilsson UR, Müller-Eberhard HJ: Isolation of b_{1F}-globulin from human serum and its characterization as the fifth component of complement. *J Exp Med* 122: 277–298, 1965
28. Vik DP, Munor-Canoves P, Chaplin DD, Tack BF: Factor H. *Curr Top Microbiol* 153: 147–160, 1989
29. Hatzakis A, Touloumi G, Karanicolas R, Karafoulidou A, Mandalaki T, Anastassopoulou C, Zhang L, Goedert JJ, Ho DD, Kostrikis LG: Effect of recent thymic emigrants on progression of HIV-1 disease. *Lancet* 355: 599–603, 2000
30. Bohling SD, Wittwer CT, King TC, Elenitoba-Johnson KSJ: Fluorescence melting curve analysis for the detection of the bcl-1/JH translocation in mantle cell lymphoma. *Lab Invest* 79: 337–345, 1999
31. Clark KR, Liu X, McGrath JP, Johnson PR: Highly purified recombinant adeno-associated virus vectors are biologically active and free of detectable helper and wild-type viruses. *Hum Gene Ther* 10: 1031–1039, 1999
32. Fink L, Seeger W, Ermert L, Hanze J, Stahl U, Grimminger F, Kummer W, Bohle RM: Real-time quantitative RT-PCR after laser-assisted cell picking. *Nat Med* 4: 1329–1333, 1998
33. Johnson MR, Wang K, Smith JB, Heslin MJ, Diasio RB: Quantitation of dihydropyrimidine dehydrogenase expression by real-time reverse transcription polymerase chain reaction. *Anal Biochem* 278: 175–184, 2000
34. Kühn S, Skerka C, Zipfel PF: Mapping of the complement regulatory domains in the human factor H-like protein 1 and factor H. *J Immunol* 155: 5663–5670, 1995
35. Ying L, Katz Y, Schlesinger M, Carmi R, Shalev H, Haider N, Beck G, Sheffield VC, Landau D: Complement factor H gene mutation associated with autosomal recessive atypical hemolytic uremic syndrome. *Am J Hum Genet* 65: 1538–1546, 1999
36. Buddles MRH, Donne RL, Richards A, Goodship J, Goodship THJ: Complement factor H gene mutation associated with autosomal recessive atypical hemolytic uremic syndrome [Letter]. *Am J Hum Genet* 66: 1721–1722, 2000
37. Skerka C, Kühn S, Günther K, Lingelbach K, Zipfel PF: A novel short consensus repeat-containing molecule is related to human complement factor H. *J Biol Chem* 268: 2904–2908, 1993
38. Schmidt BZ, Fowler NL, Hidvegi T, Perlmutter DH, Colten HR: Disruption of disulfide bonds is responsible for impaired secretion in human complement factor H deficiency. *J Biol Chem* 274: 11782–11788, 1999
39. Day DA, Tuite MF: Post-transcriptional gene regulatory mechanism in eukaryotes: An overview. *J Endocrinol* 157: 361–371, 1998
40. Fijen CA, Kuijper EJ, Te Bulte M, van de Heuvel MM, Holdrinet AC, Sim RB, Daha MR: Heterozygous and homozygous factor H deficiency states in a Dutch family. *Clin Exp Immunol* 105: 511–516, 1996
41. Sim RB, Kolble K, McAleer MA, Dominguez O, Dee VM: Genetics and deficiencies of the soluble regulatory proteins of the Complement system. *Int Rev Immunol* 10: 65–86, 1993
42. Vogt BA, Wyatt RJ, Burke BA, Simonton SC, Kashtan CE: Inherited factor H deficiency and collagen type III glomerulopathy. *Pediatr Nephrol* 9: 11–15, 1995
43. Ault BH, Schmidt BZ, Fowler NL, Kashtan CE, Ahmed AE, Vogt BA, Colten HR: Human factor H deficiency. Mutations in framework cysteine residues and block in H protein secretion and intracellular catabolism. *J Biol Chem* 272: 25168–25175, 1997
44. Sharma AK, Pangburn MK: Identification of three physically and functionally distinct binding sites for C3b in human complement factor H by deletion mutagenesis. *Proc Natl Acad Sci USA* 93: 10996–11001, 1996
45. Blackmore TK, Hellwage J, Sadlon TA, Higgs N, Zipfel PF, Ward HM, Gordon DL: Identification of the second heparin-binding domain in human complement factor H. *J Immunol* 160: 3342–3348, 1998
46. Faham S, Hileman RE, Fromm JR, Linhardt RJ, Rees DC: Heparin structure and interaction with basic fibroblast growth factor. *Science* 271: 1116–1120, 1996
47. Prodinger WM, Hellwage J, Spruth M, Dierich MP, Zipfel PF: The C-terminus of factor H: Monoclonal antibodies inhibit heparin binding and identify epitopes common to factor H and factor H-related proteins. *Biochem J* 331: 41–47, 1998
48. Meri S, Pangburn MK: Discrimination between activators and nonactivators of the alternative pathway of complement: Regulation via a sialic acid/polianion binding site on factor H. *Proc Natl Acad Sci USA* 87: 3982–3986, 1990
49. Williams DG, Peters DK, Fallows J, Petrie A, Kourilsky O, Morel-Maroger L, Cameron JS: Studies of serum complement in the hypocomplementemic nephritides. *Clin Exp Immunol* 18: 391–405, 1974
50. Tomar RH, Kolchins D: Complement and coagulation. Serum beta-Ic-beta-Ia in disseminated intravascular coagulation. *Thromb Diath Hemorrh* 27: 389–395, 1972

CHAPTER 4

Complement factor H mutations and gene polymorphisms in haemolytic uraemic syndrome: the C-257T, the A2089G and the G2881T polymorphisms are strongly associated with the disease

J. Caprioli , F. Castelletti, S. Bucchioni, P. Bettinaglio, E. Bresin, G. Pianetti, S. Gamba, S. Brioschi, E. Daina, G. Remuzzi and M. Noris, published in

Human Molecular Genetics 2003;12:3385-3395

Human Molecular Genetics, 2003, Vol. 12, No. 24 1–11
DOI: 10.1093/hmg/ddg363

Complement factor H mutations and gene polymorphisms in haemolytic uraemic syndrome: the C-257T, the A2089G and the G2881T polymorphisms are strongly associated with the disease

Jessica Caprioli[1], Federica Castelletti[1], Sara Bucchioni[1], Paola Bettinaglio[1], Elena Bresin[1], Gaia Pianetti[1], Sara Gamba[1], Simona Brioschi[1], Erica Daina[1], Giuseppe Remuzzi[1,2] and Marina Noris[1,*] for the International Registry of Recurrent and Familial HUS/TTP

[1]Mario Negri Institute for Pharmacological Research, Clinical Research Center for Rare Diseases 'Aldo e Cele Daccò', Villa Camozzi, Ranica, Italy and [2]Unit of Nephrology and Dialysis, Azienda Ospedaliera, Ospedali Riuniti di Bergamo, Italy

Received September 4, 2003; Revised and Accepted October 17, 2003

Mutations in complement factor H (HF1) gene have been reported in non-Shiga toxin-associated and diarrhoea-negative haemolytic uraemic syndrome (D$^-$HUS). We analysed the complete HF1 in 101 patients with HUS, in 32 with thrombotic thrombocytopenic purpura (TTP) and in 106 controls to evaluate the frequency of HF1 mutations, the clinical outcome in mutation and non-mutation carriers and the role of HF1 polymorphisms in the predisposition to HUS. We found 17 HF1 mutations in 33 HUS patients (16 heterozygous, one homozygous). Thirteen mutations were located in exons XXII and XXIII. No TTP patient carried HF1 mutations. The disease manifested earlier and the mortality rate was higher in mutation carriers than in non-carriers. Kidney transplants invariably failed for disease recurrences in patients with HF1 mutations, while in non-mutated patients half of the grafts were functioning after 1 year. Three HF1 polymorphic variants were strongly associated with D$^-$HUS: -257T (promoter region), 2089G (exonXIV, silent) and 2881T (963Asp, SCR16). The association was stronger in patients without HF1 mutations. Two or three disease-associated variants led to a higher risk of HUS than a single one. Analysis of available relatives of mutated patients revealed a penetrance of 50%. In 5/9 families the proband inherited the mutation from one parent and two disease-associated variants from the other, while unaffected carriers inherited the protective variants. In conclusion HF1 mutations are frequent in patients with D$^-$HUS (24%). Common polymorphisms of HF1 may contribute to D$^-$HUS manifestation in subjects with and without HF1 mutations.

INTRODUCTION

The term thrombotic microangiopathy (TMA) defines a lesion of vessel wall thickening (mainly arterioles and capillaries), intraluminal platelet thrombosis and partial or complete obstruction of the vessel lumina (1,2). Depending on whether renal or brain lesions prevail, two pathologically indistinguishable, but somehow clinically different entities have been described: the haemolytic uraemic syndrome (HUS) and the thrombotic thrombocytopenic purpura (TTP). The most common form of HUS of children, with predominant renal failure, is associated with infection by strains of *E. coli* which produce a powerful Shiga-like toxin (3). This form, also called D$^+$HUS, has an excellent prognosis. In contrast, forms of HUS non-Shiga toxin-associated and diarrhoea-negative (D$^-$HUS), have a much poorer prognosis and are often relapsing; death and end-stage renal failure are the final outcome in the majority of cases (4–6). In selected cases there is a clustering of affected

*To whom correspondence should be addressed at: Mario Negri Institute for Pharmacological Research, Clinical Research Center for Rare Diseases 'Aldo e Cele Daccò', Villa Camozzi, Via Camozzi 3, 24020 Ranica, Italy. Tel: +39 03545351; Fax: +39 0354535377; Email: noris@marionegri.it

individuals within families which is suggestive of an underlying genetic predisposition to the disease. Both autosomal dominant and autosomal recessive mode of inheritance have been recognized, with precipitating events such as pregnancy, virus-like disease or sepsis being reported in some cases (7–12). Evidences is now emerging that some of these atypical forms are associated with abnormalities of the complement system due to genetic deficiency of factor H (HF), a plasma protein that inhibits the activation of the alternative pathway of complement (13–15). After the first report showing that an area on chromosome 1q, where HF1 is mapped, segregates with the disease (16), mutations in HF1 have been described in familial but also in non-familial cases of atypical HUS, the majority of which are heterozygous and cause either single amino acid exchanges or premature translation interruption within the C-terminal domain of the protein (Short Consensus Repeat 20, SCR20) (17–21).

On the other hand evidence is available that TTP may be caused by a deficiency of ADAMTS-13, a plasma metalloprotease that cleaves ultralarge (UL) multimers of von Willebrand factor (VWF) soon after their secretion by endothelial cells (22). The deficiency of ADAMTS-13 activity may be constitutive, as in patients carrying mutations in the gene encoding for the protease (23–28), or acquired due to the presence of a circulating autoantibody (29). However the issue is far more complex since around one-third of patients with TTP do have normal ADAMTS-13 activity (29).

Taking advantage of the large number of patients referred to our international Registry of HUS and TTP, we designed a study aimed at evaluating the frequency of HF1 mutations in atypical HUS and compared the clinical phenotype of patients with and without HF1 mutations. Various polymorphisms in the promoter and in the coding regions of HF1 gene have been described which may be associated with reduced HF levels or activity. Thus a second aim of the study was to evaluate the frequency of HF1 polymorphisms in patients with HUS as compared with a control population to establish if any of them segregate with the disease. The same analyses were performed in patients with TTP to establish whether HF1 gene mutations and polymorphisms may have a role in those patients as well, or whether genetic abnormalities of HF1 were specific for the HUS phenotype.

RESULTS

Mutation screening of HF1

SSCP analysis of the complete HF1 sequence in our series of 101 patients with D$^-$HUS (familial HUS, $n=48$; recurrent HUS, $n=23$; sporadic HUS, $n=30$) and 32 patients with TTP (familial TTP, $n=7$; recurrent TTP, $n=18$; sporadic TTP, $n=7$) and in 106 controls was performed. Nine mutations were found in 11 patients with familial ($n=2$, within one family), recurrent ($n=4$) or sporadic ($n=5$) forms of D$^-$HUS (Table 1). An A305G substitution in exon II, determining an Arg60Gly change in SCR1, was found in a patient with sporadic HUS (S023#101). In exon XIX, encoding for SCR16, a G2923T (Gln950His) and a T2924C (Tyr951His) were found in two patients (S026#118 and S025#087) with sporadic HUS. A C3562G mutation (Cys1163Trp) in exon XXII, encoding for SCR19, was found in a patient with recurrent HUS (R087#134). The other mutations were located in exon XXIII encoding for SCR20, namely a G3587T determining a Glu1172Stop in two unrelated patients with sporadic and recurrent HUS (S027#022 and R063#081), a G3654A (Gly1194Asp) in two patients with familial HUS (F169#130 and F170#130), an A3666C (Glu1198Ala) in a patient with recurrent HUS (R088#152), a T3663C (Val1197Ala) in a patient with recurrent HUS (R062#056), and a C3701T (Arg1210Cys) in a patient with sporadic HUS (S013#069). The latter two mutations have been previously reported in other patients with HUS (17), while all the other mutations are new.

Table 1 and Figure 1 summarize all the mutations found in patients from our Registry, including the ones reported here and those previously published by our group (17,20). All are heterozygous mutations, with the exception of family #029, with recessive transmission. Seventy percent of the overall independent mutational events (12 out of 17) cluster in SCR20 (Table 1). Moreover three other mutations are located in SCR16 ($n=2$) and SCR19 ($n=1$), thus confirming previously reported data on the importance of the C-terminus of HF1 (17–19) to the pathogenesis of HUS.

Three mutations determine the introduction of a premature stop codon, resulting in truncated proteins at SCR 8 ($n=1$) and 20 ($n=2$), while all the others are missense mutations (Table 1).

No mutation in HF1 gene was found in patients with TTP. None of the mutations described were found in any of 106 healthy controls.

The frequency of HF1 gene mutations in our population of HUS patients (33 out of 101, including both alive and dead patients) is 33%. This frequency decreases to 24% if we consider only one patient from each family included in the study. Mutation frequency in familial forms of HUS is 46% (32% if we consider only one patient from each family), in recurrent forms 26% and in sporadic forms 17%.

Clinical and biochemical findings

The clinical data of the patients with HF1 mutations (including the new and the previously published cases from our Registry) and of the patients with no HF1 mutations are reported in Tables 2 and 3. Disease manifested earlier in patients with HF1 mutations as compared with patients carrying no HF1 mutations. However in both groups at least half of the patients developed the disease in infancy. In contrast, in all patients with TTP the onset of the disease was in adulthood. Putative triggering conditions were recognized in the majority of HUS and TTP patients, with infection being the most frequently associated condition (Table 2).

As for clinical outcome, 12 out of 33 patients with HF1 mutations (36%) died because of the disease while mortality was significantly ($P=0.016$) lower (10%) in patients without HF1 mutations, which suggests that the disease is more severe in the former than in the latter patients. Among the survivors, 57% of patients with HF1 mutation and 46% of patients without HF1 mutations were on chronic dialysis. Overall 73% of patients with HF1 mutations versus 51% of patients without mutations ($P=0.04$) died or had irreversible loss of their

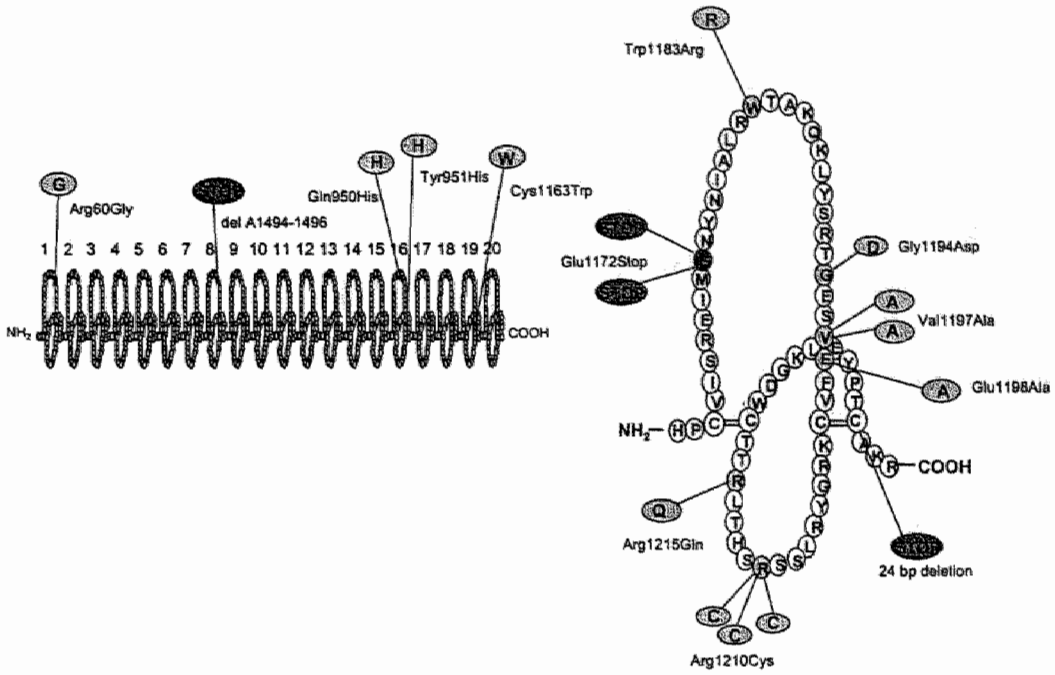


Figure 1. Summary of HF1 gene mutations in patients with D⁻HUS from our Registry. The structure of HF with the 20 SCR is shown; SCR20 is enlarged on the right. The consequence of the mutation to protein composition is indicated in the circles; each circle corresponds to an independent mutational event.

Table 1. HF1 gene mutations in patients with HUS from our Registry

Exon	Mutation	SCR	Effect	Family code	Subgroups	Patients	Unaffected carriers	Origin of the mutation
II	A305G	1	Arg60Gly	101	Sporadic	1	2/4	Paternal
XI	del A1494–1496[a]	8	Premature stop	003	Familial	2	1/11	Paternal
XIX	G2923T	16	Gln950His	118	Sporadic	1	/	Unknown
XIX	T2924C	16	Tyr951His	087	Sporadic	1	/	Unknown
XXII	C3562G	19	Cys1163Trp	134	Recurrent	1	/	Unknown
XXIII	G3587T	20	Glu1172Stop	022	Sporadic	1	/	Unknown
XXIII	G3587T	20	Glu1172Stop	081	Recurrent	1	4/8	Paternal
XXIII	T3620A[b]	20	Trp1183Arg	045	Familial	2	1/3	Paternal
XXIII	G3654A	20	Gly1194Asp	130	Familial	2	5/8	Maternal
XXIII	T3663C	20	Val1197Ala	056	Recurrent	1	/	Unknown
XXIII	T3663C[a]	20	Val1197Ala	052	Recurrent	1	1/2	Paternal
XXIII	A3666C	20	Glu1198Ala	152	Recurrent	1	/	Unknown
XXIII	C3701T[a]	20	Arg1210Cys	020	Recurrent	1	6/10	Paternal
XXIII	C3701T[a]	20	Arg1210Cys	024	Familial	3	1/3	Paternal
XXIII	C3701T	20	Arg1210Cys	069	Sporadic	1	1/1	Unknown
XXIII	G3717A[a]	20	Arg1215Gln	001	Familial	3	2/6	Paternal
XXIII	A3579T and 24 bp deletion[a]	20	Premature stop	029	Familial	10	/	Recessive

[a]Caprioli *et al.* (17).
[b]Remuzzi *et al.* (20).

Table 2. Clinical and biochemical data of patients with HUS and TTP from our Registry. The numbers of patients for whom data were available are reported in parentheses

	HUS HF1 mutation	No HF1 mutation	TTP	*P*-values (HF1mutation versus no HF1 mutation)	*P*-values (HUS versus TTP)
Disease onset					
Infancy (≤12 years)	23 (33)	34 (68)	0 (32)	0.06	<0.001
Adulthood (>12 years)	10 (33)	34 (68)	32 (32)		
Triggers					
Pregnancy	2 (29)	5 (53)	5 (29)	0.69	0.19
Drugs	2 (29)	3 (53)	4 (29)	0.82	0.19
Flu-like, gastroentheritis, other infections	13 (29)	35 (53)	11 (29)	0.06	0.06
Transplantation (post-transplant HUS)	0 (29)	3 (53)	0 (29)	0.19	0.29
Other triggers	2 (29)	2 (53)	3 (29)	0.53	0.30
Outcome					
Dialysis	12 (33)	28 (68)	0 (32)	0.64	<0.001
Kidney transplantation	5 (33)	8 (68)	0 (32)	0.63	0.03
Death	12 (33)	7 (68)	4 (32)	0.016	0.025
Relapses	20 (33)	23 (68)	13 (32)	0.01	0.84
Biochemical evaluations at remission					
Decreased C3 serum levels (≤83 mg/dl)	14 (21)	26 (51)	9 (26)	0.22	0.53
Decreased HF serum levels (≤350 mg/l)	3 (17)	1 (56)	1 (22)	0.016	0.86
Elevated HF serum levels (≥750 mg/l)	3 (17)	6 (56)	7 (22)	0.45	0.03
Moderately decreased ADAMTS13 activity (20–50%)	2 (14)	6 (58)	0 (29)	0.67	0.06
Severely decreased ADAMTS13 activity (≤20%)	0 (14)	5 (58)	17 (29)	0.25	<0.001

Table 3. Outcome of kidney transplants in patients with HUS from our Registry

	HF1 mutation	No HF1 mutation
Kidney transplant recipients	5[a]	8
Transplanted kidneys	5	11
Kidney outcome		
OK at one year	0	5*
Disease recurrency on the graft	5	2*
Acute rejection	0	4
Combined kidney and liver transplantation	2	0
Kidney outcome		
OK at one year	1[b]	0
Disease recurrency on the graft	0	0
Failure for other causes	1	0

*$P < 0.05$ versus HF1 mutation.
[a]One patient reported by Landau *et al.* (40).
[b]Reported in Remuzzi *et al.* (20).

renal function. One or more disease relapses were reported in 61 and 34% of patients with and without HF1 mutations, respectively ($P = 0.01$), and in 41% of patients with TTP.

Five patients with HF1 mutations and eight patients without mutations underwent kidney transplantation, with two patients in the latter group receiving three and two kidney grafts, respectively (Tables 2 and 3). All five grafts in patients with HF1 mutations were lost because of disease recurrence within the first year, as compared with only two of the 11 grafts in patients without HF1 mutations ($P = 0.0022$). In patients without HF1 mutations, four grafts were lost because of acute vascular rejection and the remaining five were functioning well at 1 year post transplant. Of note, two additional patients with HF1 mutations received a combined kidney and liver transplant. The kidney graft outcome at one year was very good in the first patient (20), while unfortunately the second patient died few days after surgery because of acute liver failure without evidence of recurrence of the microangiopathic process (unpublished data, Table 3).

Complement profile and ADAMTS13 activity

Samples for serum complement profile measurement were available in 72 patients with HUS (21 with HF1 mutations, 51 without HF1 mutations) and in 26 patients with TTP. Patients were studied at remission to avoid any confounding effect on complement profile due to disease activity. As shown in Table 2, decreased serum C3 levels, consistent with chronic activation of the alternative pathway of complement, were detected in 67% of D^-HUS patients carrying HF1 mutations. Mean C3 levels were moderately but significantly lower than in healthy controls (69.6 ± 18.4 versus 113.0 ± 17.9 mg/dl, $P < 0.01$). The lowest C3 levels were found in patients of family 29 (around 10 mg/l), carrying the homozygous A3579T and 24 bp deletion in exon XXIII that causes a very severe reduction of HF serum concentrations (17), in patient F039#003 (41 mg/l) with the del A1494-1496 mutation in exon XI, and in patient S023#101 (50 mg/l) with the A305G change in exon II. Of interest also 51% of HUS patients with no HF1 mutations and 35% of patients with TTP had low C3 levels after disease recovery. In contrast, C4 levels were within normal levels in the three groups of patients (not shown), thus excluding a role for the classic pathway of complement activation.

HF serum levels were lower than normal in three patients with HF1 mutations and in one patient without mutation, while three and six patients, respectively, had elevated HF serum levels.

Most patients with TTP had a severe deficiency in the plasma activity of the metalloprotease ADAMTS-13, confirming previously reported data (29), however we also found five patients with a clinical diagnosis of HUS and no HF1 mutation, showing severe ADAMTS-13 deficiency (Table 2). Two patients had familial HUS while the other three had a recurrent form of the disease with neonatal onset. The latter form, previously classified as recurrent HUS (30), has been recently redefined as Upshaw–Schulman syndrome (30). No patient had both HF1 mutations and ADAMTS-13 deficiency.

Analysis of factor H polymorphisms

HF1 gene screening in the 106 healthy controls revealed the presence of eight polymorphic variants: C-257T (promoter region), G257A in exon II (causing a Val44Ile change in SCR1), C994A in exon VII (SCR5, silent), T1277C in exon IX (causing a Tyr405His change in SCR7), G1492A in exon XI (SCR8, silent), A2089G in exon XIV (SCR11, silent), A2489-30C (intron XVI), and a G2881T in exon XIX (causing a Glu963Asp change in SCR16) (Table 4), all of which have been reported previously (19,31) with the exception of the novel variant, A2489-30C in intron XVI. The distribution of the above polymorphic variants in patients and in controls was in Hardy–Weinberg equilibrium.

Association analysis was performed in patients and healthy controls to examine whether the presence of any of the above genetic HF1 variants conferred predisposition to develop HUS or TTP. In order to avoid the bias determined by the clustering of specific variants into families, we only considered one affected subject from each family.

Comparison of allelic frequencies in patients and controls showed a strong association between HUS and the variants -257T in the promoter region, 2089G in exon XIV (SCR11) and 2881T in exon XIX (SCR16) (Table 4, $P < 0.01$ for all three polymorphisms). The association was also significant for all the three genetic variants when data from patients with HUS and no HF1 mutation were analysed separately, indicating that HF1 genetic variations may have a role in predisposing to HUS also in patients without HF1 mutations (Table 5). Similar results were obtained by analysing the distribution of the genotypes; the highest association with HUS was found for the GT + TT genotypes of the polymorphism in SCR16 (Table 6), with the highest increase in the risk of developing HUS for genotype carriers (Table 7). Mean values of HF serum concentrations were very comparable in the subgroups of patients carrying the different genotypes for the C-257T (CC, 567.3 ± 188.6 mg/l; CT, 615 ± 167 mg/l; TT, 587.3 ± 86 mg/l), the A2089G (AA, 559.1 ± 154 mg/l; AG, 628 ± 174 mg/l; GG, 594 ± 102 mg/l) and the G2881T (GG, 530.6 ± 160.9 mg/l; GT, 622 ± 164 mg/l; TT, 598 ± 89 mg/l) polymorphisms, indicating that none of the above genetic variants affect basal HF circulating levels.

A modest association was also found between HUS and the variants C994 in exon VII (SCR5) and the A2489-30 in intron XVI (Table 4). In contrast, none of the HF1 variants showed any association with TTP (Table 4).

We then tested for possible synergistic effect of the -257T, the 2089G and 2881T variants of HF1 on the predisposition to develop HUS. As shown in Table 8, we found that the combined presence of two of the above polymorphic variants on at least one allele was more strongly associated with HUS than the presence of single polymorphisms. The difference between HUS patients and controls further increased when the combined presence of three polymorphic variants was considered (HUS versus controls, P-value = 0.0000045, data not shown). Repeating the latter analysis taking into consideration only data from patients without HF1 mutations, we obtained a even lower P-value (0.000003), with respect to controls (data not shown).

The odds ratios for the presence of a couple of polymorphic variants were higher than those found for each polymorphism alone (Table 7), indicating an increased risk of developing HUS. As shown in Table 7, the simultaneous presence of the three predisposing polymorphic variants was associated with a further modest increase in the risk of HUS over the combination of two of them.

Analysis of familial transmission of mutations and polymorphisms

All available relatives of nine patients carrying HF1 mutations were also screened and unaffected carriers were detected, giving a mutation penetrance of 49% (Table 1; family 029 has been excluded from the analysis because of recessive transmission of the disease). The transmission of the mutation was from the father in eight cases and from the mother in one (Table 1; family 029 excluded). Interestingly, none of the parents carrying HF1 mutations had episodes of HUS. This could be explained either as autosomal dominant transmission with reduced penetrance or as a recessive trait. We then wanted to evaluate whether the recessive trait was determined by the presence of HF1 mutations combined with the -257T, the 2089G and the 2881T disease-associated polymorphic variants. To this purpose the parents and all available relatives from nine patients carrying HF1 mutations were analysed for the above polymorphisms and the transmitted alleles were reconstructed. In five out of nine families we found that the affected patients had inherited the allele carrying the mutation from one parent and an allele carrying at least two of the three predisposing polymorphic variants (mostly the -257T and the 2881T) from the other parent. As an example, in Figure 2 we show the pedigree and haplotype reconstruction of family 081, in which the patient (R063#081) and also four unaffected relatives carry the mutation Glu1172Stop in SCR20. The patient has inherited the mutation from the father and also received an allele carrying three disease-predisposing polymorphisms (-257T, 2089G and 2881T) from the mother. In contrast the father and all the other healthy mutation carriers within the family carry the non predisposing variants of all the three above polymorphisms and may therefore beprotected.

In addition, allele frequencies of disease-associated HF1 polymorphic variants were significantly higher in patients with HF1 mutations than in controls (Table 5), with the highest difference found for the 2881T ($P = 0.00021$). These data support the possibility that HF1 polymorphic variants may contribute to HUS development in mutation carriers.

Table 4. Allele frequencies of HF1 gene polymorphic variants occurring in controls and in patients

Exon	Mutation	SCR	Effect	Alleles	Controls	HUS	TTP	*P*-value HUS versus controls	*P*-value TTP versus controls
	C-257T	Promoter		C	73.6%	60.0%	81.0%	0.0069	0.24
				T	26.4%	40.0%	19.0%		
II	G257A	SCR1	Val44Ile	G	75.6%	80.8%	75.0%	0.37	0.93
				A	24.4%	19.2%	25.0%		
VII	C994A	SCR5	Silent	C	62.2%	78.3%	61.0%	0.01	0.87
				A	37.8%	21.7%	39.0%		
IX	T1277C	SCR7	Tyr405His	T	69.0%	78.0%	64.0%	0.38	0.57
				C	31.0%	22.0%	36.0%		
XI	G1492A	SCR8	Silent	G	55.0%	57.0%	58.0%	0.72	0.71
				A	45.0%	43.0%	42.0%		
XIV	A2089G	SCR11	Silent	A	83.0%	64.5%	90.0%	0.000052	0.19
				G	17.0%	35.5%	10.0%		
IntronXVI	A2489-30C	/	Silent	A	58.5%	74.7%	56.0%	0.037	0.74
				C	41.5%	25.3%	44.0%		
XIX	G2881T	SCR16	Glu963Asp	G	82.0%	63.0%	87.0%	0.000039	0.38
				T	18.0%	37.0%	13.0%		

DISCUSSION

In our series of 101 patients with D$^-$HUS we found 17 independent HF1 mutational events (present data and 17,20), of which seven mutations are novel. These mutations appear to be responsible for the disease in 33 of these patients. As expected, the frequency of HF1 mutation is high in cases with a familial history of HUS but rather low in sporadic cases. By contrast no patient with a diagnosis of TTP carried HF1 mutations which indicates that HF abnormalities are specifically associated with the HUS phenotype.

All are heterozygous mutations, with the exception of one homozygous mutation found in a family with recessive transmission. Of note most mutations caused single amino acid changes, while three mutations introduced premature stop codons. Although the identified mutations are spread over five exons, most of them (13 out of 17) clustered in exons XXII and XXIII and affected the most C terminus part of HF (SCR19 and 20).

In humans the alternative pathway of complement activates continuously by deposing C3b on all surfaces in contact with plasma. Amplification of the initial C3b formation and subsequent activation of the full complement system is controlled on host surfaces by regulatory proteins, some of which are membrane-bound and some fluid phase proteins (32–34). HF is the primary fluid phase regulator and is responsible for controlling spontaneous fluid phase activation as well as activation on host cells by binding to surface polyanions such as clusters of sialic acid and sulfated proteoglycans, such as heparin (32,33). Detailed structure function analysis has localized within the N-terminal SCRs 1–4 of HF the complement regulatory domains needed to prevent runaway fluid phase alternative pathway amplification (35). On the other hand the C-terminal domains of HF have numerous functions which control the regulatory activity of the N-terminal domains: there are two C3b binding sites, which raise the affinity of HF for C3b clusters on the surface of cells, and three polyanion-binding sites (35–37), which determine the contact of the molecule with host cells. Recent studies indicate that deletion of the last C-terminus SCRs of HF causes loss of the ability of the protein to control spontaneous activation of the alternative complement pathway on host cells surface (37). In addition functional studies by two independent groups (36,38) documented that five of the mutations in SCR20 reported in patients with HUS, namely Glu1172Stop, Arg1210Cys, Arg1215Gly, Trp1183Leu and Val1197Ala (36,38), cause reduced binding of the protein to surface-bound C3b, to heparin and to endothelial cells. In contrast, the mutant proteins have a normal capacity to bind fluid-phase C3b and to act as cofactors for C3b proteolysis by factor I (38). This may explain why, in patients with mutations in the C terminus of HF, C3 serum levels are in general only moderately reduced or even normal, while strong deposition of C3 (39) and C5b-9 (40) is found in glomeruli and renal arterioles.

The disease manifested earlier and was associated with a higher mortality rate in carriers of HF1 mutations than in those without HF1 mutations. However, a consistent number of patients carrying HF1 mutations had long remissions and presented late in life, suggesting that a 'second hit' is needed, at least in the heterozygous individuals. Actually, a putative triggering condition associated with HUS onset, in particular infection, was found in most patients. According to this possibility, in HUS patients, decreased availability of wild-type HF, which may be enough to control basal complement activation, upon a stimulus that activates the complement system (such as infection, cytotoxic drugs and other intercurrent stimuli), fails to efficiently restrict complement deposition on endothelial cells, leading to damage to microvasculature cell membranes and to tissue destruction.

Five patients with HF1 mutations were given a renal transplantation. In all of them the graft failed because of disease recurrence. Since HF is mostly synthesized by the liver (34), the genetic defect was not corrected by the kidney transplant and persistent HF deficiency predisposed to disease recurrence on the transplanted kidney. In an additional patient with HF1 mutation HUS recurrence was prevented by a simultaneous liver transplant, which corrected HF dysfunction (20). For still

Table 5. Allele frequencies of disease-associated HF1 polymorphic variants in patients with and without HF1 mutations and in controls

Genetic variant	Alleles	HUS HF1 mutation	HUS no HF1 mutation	Controls	*P*-value, HUS HF1 mutation versus controls	*P*-value, HUS no HF1 mutation versus controls
C-257T	C	53.2%	62.1%	73.6%	0.017	0.027
Promoter	T	46.8%	37.9%	24.6%	0.017	0.027
A2089G	A	62.5%	65.0%	84.0%	0.0065	0.00020
SCR 11	G	37.5%	35.0%	16.0%	0.0065	0.00020
G2881T	G	53.0%	65.6%	82.0%	0.00021	0.00067
SCR 16	T	47.0%	34.4%	18.0%	0.00021	0.00067

Table 6. Genotype distribution of HF1 polymorphic variants in HUS patients and in controls

Variant	Genotypes	HUS patients	Controls
C-257T[a]	CC	25	59
Promoter[a]	CT	44	38
	TT	9	9
	Total	78	106
A2089G[b]	AA	29	72
SCR11[b]	AG	40	32
	GG	7	2
	Total	76	106
G2881T[c]	GG	27	70
SCR16[c]	GT	43	34
	TT	7	2
	Total	77	106

[a]*P*-value (general) = 0.0062; *P*-value (CT + TT) = 0.0015.
[b]*P*-value (general) = 0.00016; *P*-value (AG + GG) = 0.000067.
[c]*P*-value (general) = 0.000084; *P*-value (GT + TT) = 0.000034.

Table 7. Relative risk of developing HUS in subjects carrying the polymorphic HF1 variants in the promoter, in SCR11 and in SCR16 (alone or in combination)

Genotypes	Odds ratios (95% confidence interval)	*P*-value
Promoter (TT + CT)	2.66 (1.45–4.90)	0.0015
SCR11 (GG + AG)	3.43 (1.85–6.36)	<0.0001
SCR16 (TT + GT)	3.60 (1.94–6.67)	<0.0001
Promoter + SCR11 (TT + CT) + (GG + AG)	4.22 (2.23–7.99)	<0.0001
Promoter + SCR16 (TT + CT) + (TT + GT)	3.82 (2.04–7.15)	<0.0001
SCR11 + SCR16 (GG + AG) + (TT + GT)	4.33 (2.29–8.19)	<0.0001
Promoter + SCR11 + SCR16 (TT + CT) + (GG + AG) + (TT + GT)	4.44 (2.31–8.55)	<0.0001

unknown reasons, premature liver failure, cured by a second liver graft in the above case (20) and fatal in a second one (manuscript in preparation), complicated the outcome of the combined liver and kidney transplants. Thus, in patients with HF1 mutations, a single kidney graft is contraindicated and a combined kidney and liver transplant is not recommended at the moment because of the high risk of liver graft failure. On the contrary, in patients without HF1 mutations, half of the kidney grafts were still functioning at 1 year post-transplant.

Of note, 67% of patients with HUS in our series did not have HF1 mutations. These data would suggest the existence of at least another susceptibility gene for HUS. That this gene could be involved in the regulation of the alternative pathway of complement is supported by finding of lower than normal C3 levels in more than 50% of HUS patients with no HF1 mutations.

We also evaluated the possibility that common polymorphisms of HF1 gene may contribute to development of HUS in patients without HF1 mutations. Actually, analysis of allele frequency and genotype distribution of HF1 polymorphisms in patients and controls revealed an association between HUS and three HF1 polymorphic variants. Specifically, the T allele of the C-257T, the G allele of the A2089G and the T allele of the G2881T polymorphisms were more frequent in HUS patients than in controls and odds ratio values indicate that carriers of each polymorphic variant have a higher risk of developing HUS than non-carriers. The combined presence of two and three of the above variants on at least one allele was more strongly associated with HUS than the presence of a single one. Consistently, increased odds ratios for the risk of developing HUS were found in carriers of two or three of the above polymorphisms. Interestingly, when only data from patients without HF1 mutations were considered we found a stronger association between HUS and the simultaneous presence of the three predisposing polymorphic variants than by analysing data from the overall HUS population.

The first genetic variant, namely a C-257T, is located in an NFkB responsive element within the HF1 promoter (31), which could have a role in regulating HF transcription. Although we found no difference in HF levels among patients carrying the T or the C allele when studied at remission, this polymorphic variant probably affects the efficiency of NFkB-induced HF transcription in conditions of infection or inflammation (41–43). The second polymorphism, an A2089G located in exon XIV, although silent, could theoretically influence either the efficiency of transcription or the mRNA stability or just be in linkage disequilibrium with the other two polymorphisms. Finally, we found association between the disease and the polymorphic variant G2881T, determining a Glu963Asp change in SCR16 of HF. Of interest, two of the mutations in HF1 found in our patients were also located in SCR16, a few amino acids away from the Glu963Asp polymorphism, which suggests that a functional site located in SCR16 may be crucial to protect from disease development.

The distribution of the above polymorphisms was also investigated in patients carrying HF1 mutations and in all their

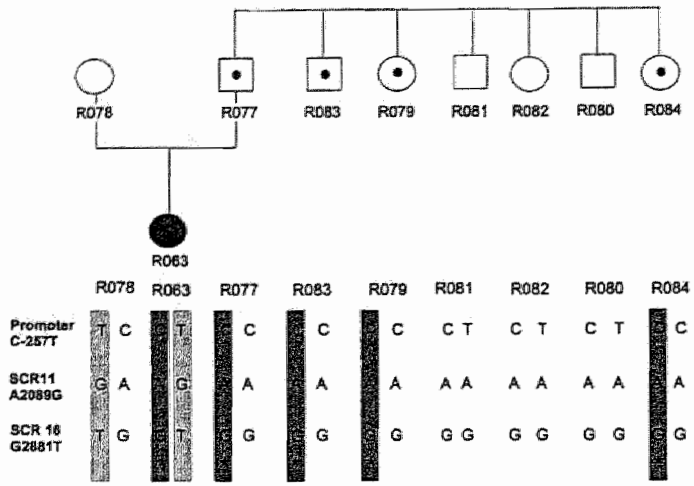


Figure 2. Pedigree of family 081 showing the segregation of the Glu1172Stop mutation. Non-affected carriers are marked with a central black circle. The C-257T in the promoter, the A2089G in SCR11 and the G2881T in SCR16 polymorphisms were analysed to determine the paternal and maternal alleles in the proband and to evaluate the segregation of the disease associated variants -257T, 2089G and 2881T in the proband and in the healthy mutation carriers. The black asterisk identifies the allele carrying the Glu1172Stop mutation.

Table 8. Distribution of combined genotypes of HF1 disease-associated polymorphic variants in HUS patients and in controls

Promoter and SCR11[a]			Promoter and SCR16[b]			SCR11 and SCR16[c]		
Genotypes	HUS patients	Controls	Genotypes	HUS patients	Controls	Genotypes	HUS patients	Controls
CCAA	19	53	CCGG	20	47	AAGG	24	67
CCGA	4	5	CCGT	5	8	AAGT	5	6
CCGG	0	0	CCTT	0	0	AATT	0	0
CTAA	9	16	CTGG	6	17	AGGG	1	5
CTGA*	34	16	CTGT*	36	21	AGGT*	38	23
CTGG*	1	0	CTTT*	1	0	AGTT*	0	2
TTAA	1	7	TTGG	1	4	GGGG	0	0
TTGA*	2	7	TTGT*	2	4	GGGT*	0	0
TTGG*	6	2	TTTT*	6	3	GGTT*	7	2
Total	76	106	Total	77	104	Total	75	105

[a] *P*-value (general) = 0.000068; *P*-value (*) = 0.0000057.
[b] *P*-value (general) = 0.0028; *P*-value (*) = 0.000019.
[c] *P*-value (general) = 0.00005; *P*-value (*) = 0.0000037.

available relatives, to evaluate whether they could discriminate between disease-affected and healthy mutation carriers in our families. Tracking the mutations back to the former generation was possibile in nine of our patients; interestingly in eight of them the mutation was inherited from the father while only in one the mutation carrier was the mother. None of the ancestors had episodes of HUS. This could be explained either as autosomal dominant transmission with reduced penetrance or as a recessive trait. The latter was confirmed in one family (029) in which the affected subjects carried an homozygous HF1 deletion in exon XXII. In an additional five families we found that the proband had inherited the allele carrying the mutation from one parent and an allele carrying at least two disease-associated HF1 polymorphic variants from the other parent, which again would reproduce a recessive trait. By contrast, all the healthy mutation carriers in the above five families had inherited the protective HF1 polymorphic variants.

In summary, this mutation study in a large population of patients demonstrates that mutations in the HF1 gene are frequent in patients with D$^-$HUS, regardless of previous familial history. In addition common polymorphic variants of HF1 gene may predispose to HUS those subjects who do not carry HF1 mutations and, in subjects with HF1 mutations, may help the full manifestation of the disease. The finding of no mutation in patients with TTP, a related thrombotic microangiopathy with mainly extra-renal involvement, calls for a specific protective role of HF from damaging effect of complement within the kidney. This interpretation is in keeping

Table 9. Sequence of the primers used for HF1 gene screening

	Sense	Antisense
Promoter	5′-caagcactgcattcttggca-3′	5′-gctagggaaatttctccgttg-3′
SCR1	5′-cactttatgcacttattttgtttttattgttt-3′	5′-acacctagttttcataaatttcacaaatg-3′
SCR2A	5′-agattccactctacattgtatgagaaa-3′	5′-atgctaacgttctgttatttttggt-3′
SCR2B	5′-tcttaattataaacctcttttcgtatggacta-3′	5′-tccttgctattacatactaattcataacttttt-3′
SCR3	5′-ggatgattttatacatacacatatttttcaca-3′	5′-ttccttagaatgaacgatgttttaaatg-3′
SCR4	5′-tttataaagatccagaaaataaaggtaacatta-3′	5′-attatgtcctggtcacagtcctttaa-3′
SCR5	5′-tttaacggatacttatttctgcattatcc-3′	5′-ttcagaattaagaaatgggtcaagatatg-3′
SCR6	5′-tgacctagaaaccctaatggaatgt-3′	5′-cacttatgtgctctcctttcttcga-3′
SCR7	5′-tgagcaaatttatgtttctcatttacttt-3′	5′-ttgccacaattaatatagatgagtcttaga-3′
SCR8	5′-atagatattgagaatgggtttatttctgaa-3′	5′-gttgagctgaccatccatctttc-3′
SCR9	5′-tcagattgtttattagatgacattagaaatga-3′	5′-cattccttaacaattcctctatataattcaaa-3′
SCR10	5′-caacctcactttattgtggcatatg-3′	5′-aaaagttggtattcaaagttctaattcttatttc-3′
SCR11	5′-tatattgtaaaacagacaatttaacc-3′	5′-atacaaaatacaaaagttttgacaag-3′
SCR12	5′-catcatgttttcacaataaacttttttg-3′	5′-ctttgttaaatgttgatttaagaagggta-3′
SCR13	5′-aactatttttatgtaatagttggtttgattcc-3′	5′-gaatacatttctgaaaacaaaatagagctt-3′
SCR14	5′-gtgataatttatgaaacagttattg-3′	5′-agagaatattaacctcatttgaaagaattatgt-3′
SCR15	5′-ttaacccttgattttcattcttcatt-3′	5′-aacagaaattgagtattcatattggcc-3′
SCR16	5′-gatgtcatagtagctcctgtattgtttattt-3′	5′-ccacttacactttgaatgaagaatatttatc-3′
SCR17	5′-gccactgggctgggc-3′	5′-tctatatatcgctattttagaatccattagatg-3′
SCR18	5′-cacttctttttttctattcagacacc-3′	5′-agaattgaatttaagcaccatcag-3′
SCR19	5′-tgaaatatcagactcatcacaga-3′	5′-atacagtgctgtgtttgcg-3′
SCR20A	5′-gttctgaataaaggtgtgcac-3′	5′-gccaaacagaagctttattc-3′
SCR20B	5′-ccccgtttacacacaaattcaa-3′	5′-ctacatagttggtttggat-3′

with experimental data showing that in an HF-deficient line of pigs, homozygous individuals die soon after birth from complement-mediated acute renal failure (44) and HF knockout mice develop membranoproliferative glomerulonephritis (45).

Further biochemical and functional studies on HF1 mutants and polymorphic variants will be required to provide deeper insights into the role of HF in the pathogenesis of HUS.

MATERIALS AND METHODS

Patients

One-hundred and one patients with diagnosis of HUS and 32 patients with diagnosis of TTP were recruited through the database of the International Registry of Recurrent and Familial HUS/TTP, a network of 60 units of Haematology and Nephrology, established on 1995, under the coordination of the Clinical Research Center for Rare Diseases 'Aldo & Cele Daccò' (Ranica, Italy).

HUS or TTP was diagnosed in all cases reported to have one or more episodes of microangiopathic haemolytic anaemia and thrombocytopenia defined on the basis of haematocrit (Ht) <30%, haemoglobin (Hb) <10 mg/dl, serum lactate dehydrogenase (LDH) >460 U/l, undetectable haptoglobin, fragmented erythrocytes in the peripheral blood smear, and platelet count <150 000/μl. Specifically, a diagnosis of HUS was made when laboratory findings of thrombotic microangiopathy were associated with acute renal failure without evidence of neurologic signs (namely signs consistent with focal or diffuse CNS ischaemia) except those attributable to uraemic or hypertensive encephalopathy. A diagnosis of TTP was made when laboratory findings of thrombotic microangiopathy occurred in patients, with or without renal involvement, in whom specific neurologic symptoms dominated the clinical picture (29).

Familial HUS or TTP was diagnosed in patients for whom at least two members of the family were affected by the disease at least 6 months apart, without evidence of a common triggering infectious agent (46). Recurrent HUS or TTP was diagnosed in patients who, with no familial history, had one or more relapses after complete and persistent remission of any sign of thrombotic microangiopathy (46). Finally, sporadic HUS or TTP was diagnosed in patients with only one reported episode of the disease. Patients with *E. coli*-associated diarrhea-positive HUS were excluded from the study.

One-hundred and six healthy controls, matched for sex and geographic origin were also studied. All patients and controls were Caucasians. Patients and controls received detailed information on the purpose and design of the study and provided informed consent according to the guidelines of the Declaration of Helsinki. The protocol was approved by the Institutional Review Board of the Mario Negri Institute.

Complement profile assessment

Serum samples were used to quantify the third (C3) and the fourth (C4) complement fractions by kinetic nephelometric measurements, and HF by radial immunodiffusion (RID) assays using commercially available kits (The Binding Site, Birmingham, UK). Serum levels of C3 and C4 below the lower limit of normal ranges (defined as mean ± SD) of the laboratories of the 'Ospedali Riuniti, Azienda Ospedaliera di Bergamo' (i.e. <83 mg/dl for C3 and <15 mg/dl for C4) were taken to indicate hypocomplementemia (17).

Measurement of ADAMTS-13 activity

ADAMTS-13 activity was measured as previously described (29) using the collagen binding assay. The protease activity was tested using pooled normal plasma as source of VWF as substrate for ADAMTS-13. Human collagen type III (3 μg/ml,

Valter Occhiena, Milano Italy) was used for the collagen binding assay. The values of the protease activity were read from a dose-response curve obtained with reference plasma pool. The lower limit of the assay was 6% of the normal protease levels (29).

Single-strand conformation polymorphism analysis and sequencing of factor H

Genomic DNA was extracted from peripheral blood leucocytes according to standard protocols (Nucleon BACC2 kit, Amersham, UK). HF1 was screened by PCR–SSCP. PCR reactions were done in a 20 µl volume containing 100 ng DNA, 15 pmol of each primer, constructed in order to avoid coamplification of factor H related genes (see Table 9), 16 nmol dNTP, 2.25 mM $MgCl_2$, 1 U *Taq* polymerase (*Taq* Gold, PE Applied Biosystems, Foster City, CA, USA), and PCR buffer. Ten minutes' denaturation at 94°C were followed by 35 PCR cycles (94°C for 45 s, 55.5°C for 30 s, and 72°C for 45 s), and by 10 min extension at 72°C. Samples were mixed with 20 µl of loading buffer, denatured at 65°C for 10 min, and electrophoresed on non denaturing 6% (62 : 1 acryl : bis) acrilamide gel in TAE buffer (pH 6.8) at 35 W for 3–5 h at 4°C. Gels were visualized by silver staining. If a polymorphism was identified, selected individuals showing the three different band patterns in SSCP were sequenced using a CEQ 2000 XL sequencer (Beckman Coulter, Berkeley, CA, USA) in order to identify the corresponding three genotypes. Those subjects were subsequently used as standards in SSCP. Subjects showing aberrant bands were also sequenced.

Statistical analysis

Differences between allele frequencies and genotype distribution of HF1 gene polymorphisms among patients with HUS, TTP and controls were analysed by the chi-square test. The odds ratios for these comparisons were also calculated. Data were expressed as mean ± SD. A P-value ≤ 0.05 was considered to be statistically significant. The two tailed t-Student test was used to compare C3, C4, C3d and HF levels in our populations.

ACKNOWLEDGEMENTS

We thank Dr Piero Ruggenenti for useful discussion, Dr Annalisa Perna for statistical advice, and Dr Miriam Galbusera for ADAMTS-13 activity measurement. Dr Paola Bettinaglio is a recipient of an Alfredo Leonardi fellowship, Dr Sara Bucchioni and Dr Simona Brioschi are recipients of fellowships from Associazione per la Ricerca sulle Malattie Rare (ARMR), through the generosity of 'AnnaVi Gioielleria', Bergamo. Dr Federica Castelletti received a fellowship in memory of Libera Dossi Grana. This work was partially supported by Comitato 30 ore per la vita, by Telethon (grant GGP02161) and by a grant from Foundation for Children with atypical HUS along with the Nando Peretti Foundation.

REFERENCES

1. Ruggenenti, P., Noris, M. and Remuzzi, G. (2001) Thrombotic microangiopathy, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura. *Kidney Int.*, **60**, 831–846.
2. Ruggenenti, P., Noris, M. and Remuzzi, G. (2003) Thrombotic microangiopathies. In Brady, H. R. and Wilcox, C. S. (eds), *Therapy in Nephrology and Hypertension: a Companion to Brenner and Rector's The Kidney*. W.B. Saunders, Philadelphia, PA.
3. Kaplan, B.S., Meyers, K.E. and Schulman, S.L. (1998) The pathogenesis and treatment of hemolytic uremic syndrome. *J. Am. Soc. Nephrol.*, **9**, 1126–1133.
4. Fitzpatrick, M.M., Walters, M.D.S. and Trompeter, R.S. (1993) Atypical (non-diarrhea-associated) hemolytic-uremic syndrome in childhood. *J. Pediatr.*, **122**, 532–537.
5. Proesmans, W. (1996) Typical and atypical hemolytic uremic syndrome. *Kidney Blood Press. Res.*, **19**, 205–208.
6. Neuhaus, T.J., Calonder, S. and Leumann, E.P. (1997) Heterogeneity of atypical hemolytic uremic syndrome. *Arch. Dis. Child.*, **76**, 518–521.
7. Kaplan, B.S. and Kaplan, P. (1992) *Hemolytic Uremic Syndrome in Families*. Marcel Dekker, New York.
8. Berns, J.S., Kaplan, B.S., Mackow, R.C. and Hefter, L.G. (1992) Inherited hemolytic uremic syndrome in adults. *Am. J. Kidney Dis.*, **19**, 331–334.
9. Kirchner, K.A., Smith, R.M., Gockerman, J.P. and Luke, R.G. (1982) Hereditary thrombotic thrombocytopenic purpura: microangiopathic hemolytic anemia, thrombocytopenia, and renal insufficiency occurring in consecutive generations. *Nephron*, **30**, 28–30.
10. Bergstein, J., Michael, A. Jr, Kjellstrand, C., Simmons, R. and Najarian, J. (1974) Hemolytic–uremic syndrome in adult sisters. *Transplantation*, **17**, 487–489.
11. Pirson, Y., Lefebvre, C., Arnout, C. and de Strihou, C.Y. (1997) Hemolytic uremic syndrome in three adult siblings: a familial study and evolution. *Clin. Nephrol.*, **28**, 250–255.
12. Carreras, L., Romero, R., Requesens, C., Oliver, A.J., Carrera, M., Clavo, M. and Alsina, J. (1981) Familial hypocomplementemic hemolytic uremic syndrome with HLA-A3, B7 haplotype. *J. Am. Med. Assoc.*, **245**, 602–604.
13. Pichette, V., Quérin, S., Schürch, W., Brun, G., Lehner-Netsch, G. and Delaghe, J-M. (1994) Familial hemolytic–uremic syndrome and homozygous factor H deficiency. *Am. J. Kidney Dis.*, **26**, 936–941.
14. Thompson, R.A. and Winterborn, M.H. (1981) Hypocomplementaemia due to a genetic deficiency of α1H globulin. *Clin. Exp. Immunol.*, **46**, 110–119. Q2
15. Rougier, N., Kazatchkine, M.D., Rougier, J.-P., Fremeaux-Bacchi, V., Blouin, J., Deschenes, G., Soto, B., Baudouin, V., Pautard, B., Proesmans, W. *et al.* (1998) Human complement factor H deficiency associated with hemolytic uremic syndrome. *J. Am. Soc. Nephrol.*, **9**, 2318–2326.
16. Warwicker, P., Goodship, T.H.J., Donne, R.L., Pirson, Y., Nicholls, A., Ward, R.M., Turnpenny, P. and Goodship, J.A. (1998) Genetic studies into inherited and sporadic hemolytic uraemic syndrome. *Kidney Int.*, **53**, 836–844.
17. Caprioli, J., Bettinaglio, P., Zipfel, P.F., Amadei, B., Daina, E., Gamba, S., Skerka, C., Marziliano, N., Remuzzi, G. and Noris, M. (2001) The molecular basis of familial hemolytic uremic syndrome: mutation analysis of factor H gene reveals a hot spot in short consensus repeat 20. *J. Am. Soc. Nephrol.*, **12**, 297–307.
18. Richard, A., Buddles, M.R., Donne, R.L., Kaplan, B.S., Kirk, E., Venning, M.C., Tielemans, C.L., Goodship, J.A. and Goodship, T.H.J. (2001) Factor H mutations in hemolitic uremic syndrome cluster in exons 18–20, a domain important for host cell recognition. *Am. J. Hum. Genet.*, **68**, 485–490.
19. Perez-Caballero, D., Gonzalez-Rubio, C., Gallardo, M.E., Vera, M., Lopez-Trascasa, M., Rodriguez de Cordoba, S. and Sanchez-Corral, P. (2001) Clustering of missense mutations in the C-terminal region of factor H in atypical hemolytic uremic syndrome. *Am. J. Hum. Genet.*, **68**, 478–484.
20. Remuzzi, G., Ruggenenti, P., Codazzi, D., Noris, M., Caprioli, J., Locatelli, G. and Gridelli, B. (2002) Combined kidney and liver transplantation for familial haemolytic uraemic syndrome. *Lancet*, **359**, 1671–1672.
21. Neumann H.P., Salzmann M., Bohnert-Iwan B., Mannuelian T., Skerka C., Lenka D., Bender B.U., Cybulla M., Riegler P., Konigsrainer A. *et al.* (2003) Haemolytic uraemic syndrome and mutations of the factor H gene: a registry-based study in German speaking countries. *J. Med. Genet.*, **40**, 676–681.

22. Furlan, M., Robles, R. and Lammle, B. (1996) Partial purification and characterization of a protease from human plasma cleaving von Willebrand factor to fragments produced by *in vivo* proteolysis. *Blood*, **87**, 4223–4234.
23. Levy, G.G., Nichols, W.C., Lian, E.C., Foroud, T., McClintick, J.N., McGee, B.M., Yang, A.Y., Siemieniac, D.R., Strak, K.R., Gruppo, R. *et al.* (2001) Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. *Nature*, **413**, 475–476.
24. Kokame, K., Matsumoto, M. and Soejima, K. (2002) Mutations and common polymorphisms in ADAMTS13 gene responsible for von Willebrand factor-cleaving protease activity. *Proc. Natl Acad. Sci. USA*, **99**, 11902–11907.
25. Antoine, G., Zimmermann, K., Plaimauer, B., Grillowitzer, M., Studt, J-D., Lammle, B. and Scheiflinger, F. (2003) ADAMTS13 gene defects in two brothers with constitutional thrombotic thrombocytopenic purpura and normalization of von Willebrand factor-cleaving protease activity by recombinant human ADAMTS13. *Br. J. Haematol.*, **120**, 821–824.
26. Savasan, S., Lee, S-K., Ginsburg, D. and Tsai, H-M. (2003) ADAMTS13 gene mutation in congenital thrombotic thrombocytopenic purpura with previously reported normal VWF cleaving protease activity. *Blood*, **101**, 4449–4451.
27. Schneppenheim, R., Budde, U., Oyen, F., Angerhaus, D., Aumann, V., Drewke, E., Hassenpflug, W., Haberle, J., Kentouche, K., Kohne, E. *et al.* (2003) von Willebrand factor cleaving protease and ADAMTS13 mutations in childhood TTP. *Blood*, **101**, 1845–1850.
28. Assink, K., Schiphorst, R., Allford, S., Karpman, D., Etzioni, A., Brichard, B., van de Kar, N., Monnens, L. and van den Heuvel, L. (2003) Mutation analysis and clinical implications of von Willebrand factor-cleaving protease deficiency. *Kidney Int.*, **63**, 1995–1999.
29. Remuzzi, G., Galbusera, M., Noris, M., Canciani, M.T., Daina, E., Bresin, E., Contaretti, S., Caprioli, J., Gamba, S., Ruggenenti, P. *et al.* (2002) von Willebrand factor cleaving protease (ADAMTS13) is deficient in recurrent and familial thrombotic thrombocytopenic purpura and hemolytic uremic syndrome. *Blood*, **100**, 778–785.
30. Veyradier, A., Obert, B., Haddad, E., Cloarec, S., Nivet, H., Foulard, M., Lesure, F., Delattre, P., Lakhdari, M., Meyer, D. *et al.* (2003) Severe deficiency of the specific von Willebrand factor-cleaving protease (ADAMTS13) activity in a subgroup of children with atypical hemolytic-uremic syndrome. *J. Pediatr.*, **142**, 310–317.
31. Warwicker, P., Goodship, T.H.J. and Goodship, J.A. (1997) Three new polymorphisms in the human complement factor H gene and promoter region. *Immunogenetics*, **46**, 437–438.
32. Meri, S. and Pangburn, M.K. (1990) Discrimination between activators and nonactivators of the alternative pathway of complement: regulation via a sialic acid/polianion binding site on factor H. *Proc. Natl Acad. Sci. USA*, **87**, 3982–3986.
33. Kazatchkine, M.D., Fearon, D.T. and Austen, K.F. (1979) Human alternative complement pathway: membrane-associated sialic acid regulates the competiton between B and BIH for cell-bound C3b. *J. Immunol.*, **122**, 75.
34. Taylor, C.M. (2001) Complement factor H and the haemolytic uraemic syndrome. *Lancet*, **358**, 1200–1202.
35. Zipfel, P.F. and Skerka, C. (1994) Complement factor H and related proteins: an expanding family of complement-regulatory proteins? *Immunol. Today*, **15**, 121–126.
36. Manuelian, T., Hellwage, J., Meri, S., Caprioli, J., Noris, M., Heinen, S., Jozsi, M., Neumann, H.P.H. and Remuzzi, G. (2003) Mutations in factor H reduce binding affinity to C3b and heparin and surface attachment to endothelial cells in hemolytic uremic syndrome. *J. Clin. Invest.*, **111**, 1181–1190.
37. Pangburn, M.K. (2002) Cutting edge: localization of the host recognition funtions of complement factor H at the carboxyl-terminal: implications for hemolytic uremic syndrome. *J. Immunol.*, **169**, 4702–4706.
38. Sanchez-Corral, P., Perez-Caballero, D., Huarte, O., Simckes, A.M., Goicoechea, E., Lopez-Trascasa, M. and Rodriguez de Cordoba, S. (2002) Structural and functional characterization of factor H mutations associated with atypical hemolytic uremic syndrome. *Am. J. Hum. Genet.*, **71**, 1285–1295.
39. Noris, M., Casiraghi, F., Ruiz Torres, M.P., Caprioli, J., Galbusera, M. and Remuzzi, G. (2001) Serum from patients with recurrent HUS and TTP induces endothelial damage and thrombus formation: role of complememt. *Mol. Immunol.*, **38**, 113–114 (Abstract).
40. Landau, D., Shalev, H., Levy-Finer, G., Polonsky, A., Segev, Y. and Katchko, L. (2001) Familial hemolytic uremic syndrome associated with complement factor H deficiency. *J. Pediatr.*, **138**, 412–417.
41. Azevedo-Martins, A.K., Lortz, S., Lenzen, S., Curi, R., Eizirik, D.L. and Tiedge, M. (2003) Improvement of the mitochondrial antioxidant defense status prevents cytokine-induced nuclear factor-kappaB activation in insulin-producing cells. *Diabetes*, **52**, 93–101.
42. Dawson, S.J., Wiman, B., Hamsten, A., Green, F., Humphries, S. and Henney, A.M. (1993) The two allele sequences of a common polymorphism in the promoter of the plasminogen activator inhibitor-1 (PAI-1) gene respond differently to interleukin-1 in HepG2 cells. *J. Biol. Chem.*, **168**, 10739–10745.
43. Friese, M.A., Hellwage, J., Jokiranta, T.S., Meri, S., Peter, H.H., Eibel, H. and Zipfel, P.F. (1999) FHL-1/reconectin and factor H: two human complement regulators which are encoded by the same gene are differently expressed and regulated. *Mol. Immunol.*, **36**, 809–818.
44. Hegasy, G., Manuelian, T., Hogasen, K., Jansen, J.H. and Zipfel, P.F. (2002) The molecular basis for hereditary porcine membranoproliferative glomerulonephritis type II. *Am. J. Pathol.*, **161**, 2027–2034.
45. Pickering, M.C., Cook, H.T., Warren, J., Bygrave, A.E., Moss, J., Walport, M.J. and Botto, M. (2002) Uncontrolled C3 activation causes membranoproliferative glomerulonephritis in mice deficient in complement factor H. *Nat. Genet.*, **31**, 424–428.
46. Noris, M., Ruggenenti, P., Perna, A., Orisio, S., Caprioli, J., Skerka, C., Vasile, B., Zipfel, P.F., Remuzzi, G. on behalf of the Italian Registry of Familial and Recurrent HUS/TTP (1999) Hypocomplementemia discloses genetic predisposition to hemolytic uremic syndrome and thrombotic thrombocytopenic purpura: role of factor H abnormalities. *J. Am. Soc. Nephrol.*, **10**, 281–293.

CHAPTER 5

Complement factor H mutation in familial thrombotic thrombocytopenic purpura with ADAMTS13 deficiency and renal involvement

M. Noris, S. Bucchioni, M. Galbusera, R. Donadelli, E. Bresin, J. Caprioli ,

F. Castelletti, S. Brioschi, F. Scheiflinger and G. Remuzzi, published in

Journal of the American Society of Nephrology 2005;16:1177-1183

Complement Factor H Mutation in Familial Thrombotic Thrombocytopenic Purpura with ADAMTS13 Deficiency and Renal Involvement

Marina Noris,* Sara Bucchioni,* Miriam Galbusera,* Roberta Donadelli,* Elena Bresin,* Federica Castelletti,* Jessica Caprioli,* Simona Brioschi,* Friedrich Scheiflinger,† and Giuseppe Remuzzi;*‡ for the International Registry of Recurrent and Familial HUS/TTP

**Mario Negri Institute for Pharmacological Research, Clinical Research Center for Rare Diseases, Aldo e Cele Daccò, Villa Camozzi-Ranica; †Baxter BioScience, Biomedical Research Center, Orth/Donau, Austria; and ‡Department of Nephrology and Dialysis, Azienda Ospedaliera, Ospedali Riuniti di Bergamo, Bergamo, Italy*

Thrombotic thrombocytopenic purpura is a rare disorder of small vessels that is associated with deficiency of the von Willebrand factor–cleaving protease ADAMTS13, which favors platelet adhesion and aggregation in the microcirculation. The disease manifests mainly with central nervous system symptoms, but cases of renal insufficiency have been reported. Presented are findings of the genetic basis of phenotype heterogeneity in thrombotic thrombocytopenic purpura in two sisters within one family. The patients had ADAMTS13 deficiency as a result of two heterozygous mutations (causing V88M and G1239V changes). In addition, a heterozygous mutation (causing an S890I change) in factor H of complement was found in the patient who developed chronic renal failure but not in her sister, who presented with exclusive neurologic symptoms.

J Am Soc Nephrol 16: 1177–1183, 2005. doi: 10.1681/ASN.2005010086

Thrombotic thrombocytopenic purpura (TTP) is a disease of small vessels characterized by anemia that is caused by erythrocyte fragmentation in the microcirculation and thrombocytopenia that is caused by intravascular thrombi of aggregated platelets (1). Recent studies provided substantial evidence that 70 to 80% of cases of TTP are triggered by a deficiency of ADAMTS13 (2–4), a plasma metalloprotease that cleaves von Willebrand factor multimers soon after their secretion by endothelial cells (1,5–7). ADAMTS13 deficiency can be constitutive, as a result of homozygous or double heterozygous mutations in the corresponding gene (8–13), or acquired, as a result of the presence of circulating inhibitory antibodies (1,3,4,14–20).

TTP manifests mainly with central nervous system symptoms, but cases of renal insufficiency have been reported (1). In rare cases, renal involvement is severe enough to cause end-stage renal failure (1,21–25). Those patients' clinical manifestations are difficult to distinguish from those of hemolytic uremic syndrome (HUS), a form of thrombotic microangiopathy characterized by predominant renal involvement, often with renal failure (1,20,26). This difficulty has given rise to a heated debate on whether a severe deficiency of ADAMTS13 activity is enough to distinguish TTP from HUS (27,28).

Here we present findings of the genetic basis of phenotype heterogeneity in patients with congenital ADAMTS13 deficiency. We studied a family with two affected sisters, one who presented with exclusive neurologic symptoms and the other one with severe renal involvement that required chronic dialysis. These diverse clinical manifestations suggested to us that the genetic background could be different.

Materials and Methods

Patients

A woman, now 60 yr old (F48), and her younger sister (F45, died in 2002 at the age of 55 yr) were referred to our International Registry of Recurrent and Familial HUS/TTP in 1996 because of history of recurrent and familial thrombotic microangiopathy. The youngest brother died at the age of 15 yr of leukemia. The other four siblings (three male and one female) all seem to be healthy and have no sign of thrombotic microangiopathy. The two patients had their first episode of the disease at the age of 22 (F45) and 23 yr (F48), respectively, during their first pregnancy and subsequently experienced at least 10 disease relapses concomitant with precipitating events such as pregnancy and spontaneous abortion in the first to second trimester or infection.

In patient F48, neurologic symptoms (dysarthria, dyslalia, aphasia, and facial paralysis) were dominant. Renal function always remained normal, and urine was always negative for proteinuria and hematuria. Remission was achieved with plasma infusions, antiplatelet agents, and anticoagulants, and at present, she is in relatively healthy condition.

In patient F45, the course of the disease was more severe. After a first mild episode of hemolytic anemia and thrombocytopenia during the third month of her first pregnancy (recovered after spontaneous abortion), she had a severe relapse at the age of 23 yr, during the fifth month of her second pregnancy. This was accompanied by confusion, psychomotor agitation, and also coma. She received blood transfusions, steroids, and anticonvulsants. Remission was achieved after spontane-

Published online ahead of print. Publication date available at www.jasn.org.

Address correspondence to: Dr. Giuseppe Remuzzi, Mario Negri Institute for Pharmacological Research, Via Gavazzeni 11, 24125 Bergamo, Italy. Phone: +39-035-319-888; Fax: +39-035-319-331; E-mail: gremuzzi@marionegri.it

ISSN: 1046-6673/1605-1177

ous abortion. After two other spontaneous abortions, each associated with disease relapses, she underwent salpingostomy. Nevertheless, she continued to have relapses, triggered by infection and associated with neurologic signs and acute renal insufficiency. Transient improvement was achieved by plasma infusions, but then renal function progressively deteriorated. At the age of 44 yr, the patient started chronic dialysis. She died at the age of 55 yr because of a cerebrovascular event.

The two patients and all of their available relatives were screened for biochemical and genetic abnormalities of both ADAMTS13 and complement regulatory proteins. One hundred healthy volunteers were also studied as control subjects.

All participants received detailed information on the purposes and design of the study and provided informed written consent, according to the guidelines of the Declaration of Helsinki. The protocol was approved by the institutional review board of the "Mario Negri" Institute for Pharmacological Research.

ADAMTS13 Activity and Antigen

ADAMTS13 activity was measured using the collagen binding assay (20). The protease activity was tested using pooled normal plasma as the source of von Willebrand factor as substrate for ADAMTS13. Human collagen type III (3 μg/ml; Valter Occhiena, Milano, Italy) was used for the collagen binding assay. The values of the protease activity were read from a dose-response curve obtained with reference plasma pool. The lower limit of the assay was 6% of the normal protease levels (20). The presence of ADAMTS13 inhibitory activity was assayed by testing ADAMTS13 activity in mixtures of test plasma and normal pooled plasma at different dilutions (20).

Plasma ADAMTS13 antigen levels were evaluated with an ELISA assay using polyclonal rabbit anti-human ADAMTS13 antibodies. Recombinant human ADAMTS13 was used as standard (29).

Complement Profile

Serum C3 and C4 concentrations were measured by nephelometry. Factor H serum concentrations were assayed by radial immunodiffusion (The Binding Site, Birmingham, UK) (30).

Microsatellite Polymorphism Genotyping

Genomic DNA was extracted either from peripheral blood leukocytes or directly from whole blood according to standard protocols (Nucleon BACC2 kit; Amersham, Little Chalfont, UK). For linkage analysis, we used microsatellite markers flanking complement factor H and membrane co-factor protein (MCP) (31) (chromosome 1q32: D1S240, D1S202, D1S412, D1S2816, D1S413, D1S456, D1S2796, D1S2692) and ADAMTS13 (8) (chromosome 9q34: D9S1847, D9S164, D9S1818, D9S1826, D9S158, D9S1838) genes, identified using the Genome Data Base. Primers were synthesized by Sigma (Sigma-Aldrich, Haverhill, UK). For each PCR reaction, we used 100 ng of DNA in 20-μl final volume that contained 15 pmol of each primer, 16 nmol of dNTP, 2.25 mM $MgCl_2$, 1 U of Taqpolymerase (TaqGold; PE Applied Biosystems, Foster City, CA), and PCR buffer. PCR was performed as follows: 10-min denaturation at 94°C, followed by 35 PCR cycles (94°C for 45 s, 55.5 to 57°C for 45 s, and 72°C for 1 min), and by 10-min extension at 72°C. Amplified DNA samples were mixed with an equal volume of loading buffer, denatured at 75°C for 5 min, and electrophoresed on denaturing 6% acrylamide gel (19:1, acrylamide:bis-acrylamide) in TBE buffer, at 55 W for 2 to 4 h at room temperature. Gels were visualized by silver staining.

Screening for Mutations

Factor H and MCP genes were screened by PCR–single-strand conformation polymorphism (31,32). PCR reactions were done in 20-μl volume that contained 100 ng of DNA, 15 pmol of each primer (Table 1) (31,32), constructed to avoid co-amplification of factor H–related genes, 16 nmol of dNTP, 2.25 mM $MgCl_2$, 1 U of Taqpolymerase, and PCR buffer. Ten-minute denaturation at 94°C was followed by 35 PCR cycles: 45 s at 94°C, 45 s at 55.5 to 57°C, 1 min at 72°C, and a 10-min step at 72°C. Samples were mixed with 20 μl of loading buffer, denatured at 65°C for 10 min, and electrophoresed on nondenaturing 6% (62:1 acrylamide:bis-acrylamide) gel in TAE buffer (pH 6.8) at 35 W for 3 to 5 h at 4°C. Gels were visualized by silver staining. Patients who showed aberrant bands were sequenced.

The complete coding sequence and intronic boundaries (Table 1) of ADAMTS13 gene were analyzed by direct sequencing after purification from agarose gel (1% in TBE) of PCR products (kit QUIAEXII; Qiagen, Hieden, Germany). PCR reactions were performed as above. Amplified DNA was sequenced on both strands using a CEQ8000 XL sequencer (Beckman Coulter, Berkley, CA), following standard protocols. To exclude that mutations were rare polymorphisms, single-strand conformation polymorphism was performed as above on DNA from patients and 100 control subjects.

Results and Discussion

ADAMTS13 activity was <6% (detection limit of the assay) in the two sisters with a diagnosis of TTP. Anti-ADAMTS13 inhibitors were not found, thus excluding an acquired deficiency. It is interesting that complete ADAMTS13 deficiency was also found in a younger brother (F50), even though he had never had any episode of thrombotic microangiopathy. Microsatellite polymorphism genotyping, using polymorphic markers on chromosome 9q34 flanking ADAMTS13 gene, showed a straight correlation between ADAMTS13 activity and haplotype data: The three subjects with complete ADAMTS13 deficiency shared both alleles, whereas subjects with half normal levels shared one allele (Figure 1A). By direct sequencing, a heterozygous G323A missense mutation located in exon 3, which causes a V88M change in the metalloprotease domain, was found in the patients and in their healthy brother with protease deficiency (Figure 2A). In the same subjects, we also found a second heterozygous mutation, a G3777T in exon 27, causing a G1239V change in the first ADAMTS13 CUB domain (8). Neither mutation was found in any of 100 unrelated healthy subjects.

In this family, ADAMTS13 antigen levels paralleled protease activity. The three subjects who carried the two mutations had undetectable ADAMTS13 activity and <10% normal mean plasma antigen levels as measured by ELISA (Figure 1A). These results are consistent with published data showing that most ADAMTS13 missense mutations that are found in patients with TTP result in impaired secretion of the protein (33). Subjects F49 and F51 with a single heterozygous mutation, the G1239V and the V88M, respectively, had approximately half normal protease activity and antigen levels. However, subject F46 without mutation had normal protease activity and antigen levels (Figure 1A).

It has been proposed that a severe deficiency of ADAMTS13 is a beacon for patients with a specific form of thrombotic microangiopathy, labeled as TTP, and could also help in tailoring treatment (27). However, evidence is emerging that different clinical presentations may reflect complex underlying ge-

Table 1. Primers used for ADAMTS13 and factor H mutation screening[a]

	Sense (5′-3′)	Antisense (5′-3′)
ADAMTS13		
exon 1	CCCTGAACTGCAACCATCTT	CAAACCCCAAAGCTGATGTA
exon 2	TCGGTCTCCCCAAGTGTTAG	AACAGGGTTGACAGCAGCTT
exon 3	TCTAGAACCATCGCCCTCTG	CCGAGCCATTCTACCTGAGT
exon 4	ACATGCTGGTGGAGTAGCCTCT	GATGGAGATGCGATGACTTGG
exon 5–6	ACGGGCTAGTCATAGGGTTG	TACAAGGACCCACTGCTTGC
exon 7	GCTGGCGCTGCGGCACTAGGG	GTTGGACGGAGGGGTGGGTTG
exon 8	ACTCCTCCGTCCCGCCTGGTG	GGCCCAGTCAAACAAAAATGT
exon 9	TCTGGGAGGGACAGTTAAGG	TACTGGTCCTGCCTCCTGAC
exon 10–11	GGGTACCCTATGGGTGAGTT	CCTGGTGTGAACCACAGATG
exon 12	GCACTTTTGTCACCCCAGTT	CCAGAGCCTGAACCACTTTG
exon 13–14	CCCAGATGCAAAGGATGAAG	ATCCAGGGCTGAGTGAGTGT
exon 15	TTTTTCCCGACCAGCTAAGA	TCAGAAGTGAGGGCATCTTG
exon 16	CCGGGAAGGAGAGTCACTG	CCCTGTAAGTGACCGCTGA
exon 17–18	GTGATTGCTTGCTGAACGAA	CAGTGTCCTCACCTGCAGAA
exon 19	GAACACCTGGAGAGGCTAGG	ACTTACAACCGCCAGGTGAC
exon 20	GAACCTGCTGGCTGATGAAT	GGATGGTGTTCTTGCTCTGG
exon 21	CACACACGCCACTTCCTG	CCACGTGTTCCCATSTSGTCTG
exon 22	CCATGCGGGCCTTATGTGCTA	TCTGGGTTGCAGTCCTCAAAG
exon 23	TCCCAGCTTCCTGTCTCTTC	TCTCCTGATTCAGCTTTCCAA
exon 24	AGTACACGTGGGTGGAGAGG	CTTTCAGGGGACACGATGAG
exon 25	TTAACTGCCTCCCAGCTTCT	CTTTGCCAGGGAGAAAGAGG
exon 26	CCTCCTGGTCTCCTTCCTCAGCTTGG	TGCCAGGAATGGGGCATGCAGCGTC
exon 27	AGCACCTTGAGCCAACAGGA	CTAGACATACCCGCACTGCAGT
exon 28	CTTGAACTCGGCTCAGTCTACCCTG	TCCCTGGCACGTGCAGACTGA
exon 29	GTTGTCCTGGCCTCTGGCA	GGGTCCCCTAGCCCTGC
Factor H		
promoter	CAAGCACTGCATTCTTGGCA	GCTAGGGAAATTCTCCGTTG
SCR1	CACTTTATGCACTTATTTTGTTTTTATTGTTT	ACACCTAGTTTTCATAAATTTCACAAATG
SCR2A	AGATTCCACTCTACATTGTATGAGAAA	ATGCTAACGTTCTGTTATTTTTTGGT
SCR2B	TCTTAATTATAAACCTCTTTTCGTATGGACTA	TCCTTGCTATTACATACTAATTCATAACTTTTT
SCR3	GGATGATTTTATACATACACATATTTTTCACA	TTCCTTAGAATGAACGATGTTTTAAATG
SCR4	TTTATAAAGATCCAGAAAATAAAGGTAACATTA	ATTATGTCCTGGTCACAGTCCTTTAA
SCR5	TTTAACGGATACTTATTTCTGCATTATCC	TTCAGAATTAAGAAATGGGTCAAGATATG
SCR6	TGACCTAGAAACCCTAATGGAATGT	CACTTATGTGCTCTCCTTTCTTCGA
SCR7	TGAGCAAATTTATGTTTCTCATTTACTTT	TTGCCACAATTAATATAGATGAGTCTTAGA
SCR8	ATAGATATTGAGAATGGGTTTATTTCTGAA	GTTGAGCTGACCATCCATCTTTC
SCR9	TCAGATTGTTTATTAGATGACATTAGAAATGA	CATTCCTTAACAATTCCTCTATATAATTCAAA
SCR10	CAACCTCACTTTATTGTGGCATATG	AAAAGTTGGTATTCAAAGTTCTAATTCTTATTTC
SCR11	TATATTGTAAAACAGACAATTTAACC	ATACAAAATACAAAAGTTTTGACAAG
SCR12	CATCATGTTTTCACAATAAACTTTTTTTG	CTTTGTTAAATGTTGATTTAAGAAGGGTA
SCR13	AACTATTTTTATGTAATAGTTGGTTTGATTCC	GAATACATTTCTGAAAACAAAATAAGAGCTT
SCR14	GTGATAATTTATGAAACAGTTATTG	AGAGAATATTAACCTCATTTGAAAGAATTATGT
SCR15	TTAACCCTTTGATTTTCATTCTTCATT	AACAGAAATTGAGTATTCATATTGGCC
SCR16	GATGTCATAGTAGCTCCTGTATTGTTTATTT	CCACTTACACTTTGAATGAAGAATATTTATC
SCR17	GCCACTGGGCTGGGC	TCTATATATCGCTATTTTAGAATCCATTAGATG
SCR18	CACTTCTTTTTTTTCTATTCAGACACC	AGAATTGAATTTTAAGCACCATCAG
SCR19	TGAAATATCAGACTCATCACAGA	ATACAGTGCTGTGTTTGCG
SCR20A	GTTCTGAATAAAGGTGTGCAC	GCCAAACAGAAGCTTTATTC
SCR20B	CCCCGTTTACACACAAATTCAA	CTACATAGTTGGTTTGGAT

[a]SCR, short consensus repeat.

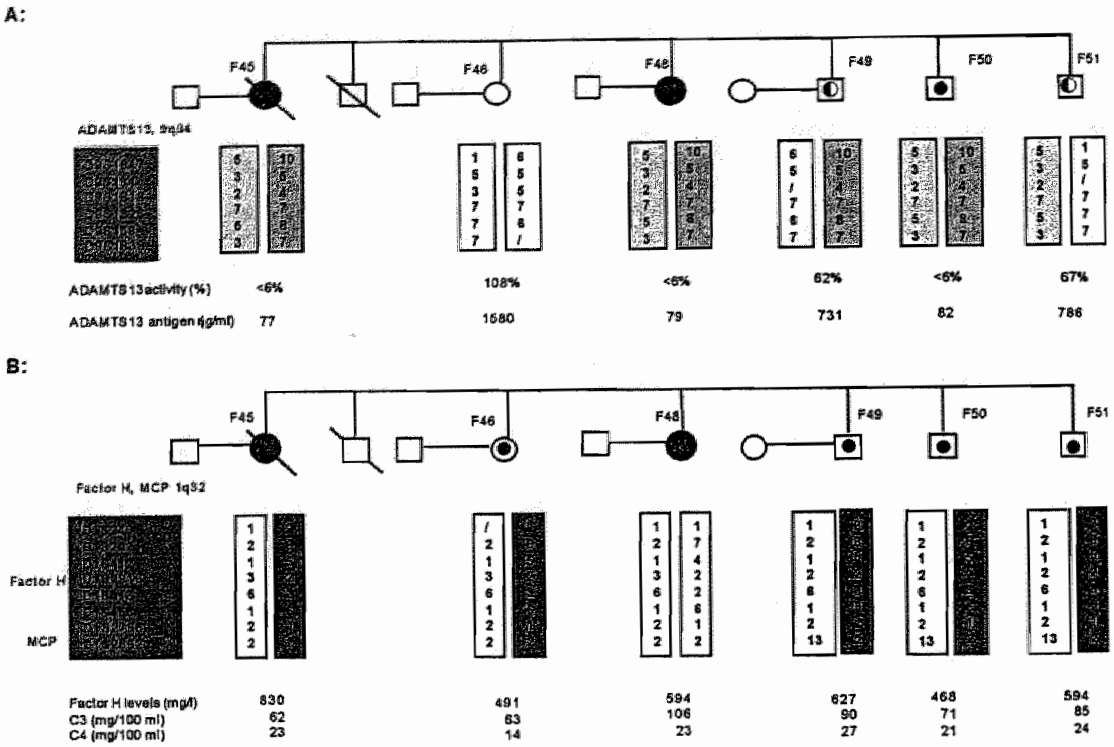


Figure 1. Linkage analysis for chromosome 9q34 (ADAMTS13; A) and chromosome 1q32 (factor H and membrane co-factor protein [MCP]; B) in the pedigree. Circles indicate female individuals; squares indicate male individuals. Affected individuals are indicated by solid symbols, and carriers are indicated by symbols with dots. (A) Six markers (pink square) flanking the ADAMTS13 gene were selected from Genome Data Base for haplotype analysis. Chromosomes that carry the mutations are colored (V88M, yellow; G1239V, green), and normal chromosomes are white. Plasma ADAMTS13 activity and antigen levels are shown below the genotypes. Normal levels, ADAMTS13 activity (range, 50 to 150%); ADAMTS13 antigen (range, 680-1350 ng/ml). (B) Eight markers (blue square) flanking the factor H and MCP genes were selected from the Genome Data Base for haplotype analysis. Chromosomes that carry the mutation are red, and normal chromosomes are white. Serum factor H, C3, and C4 levels are shown below the genotypes. Normal levels: factor H, 350 to 750 mg/L; C3, 83 to 177 mg/100 ml; C4, 15 to 45 mg/100 ml. In the two patients, both ADAMTS13 and complement parameters were measured at remission.

netic abnormalities, because ADAMTS13 deficiency results in a very heterogeneous pattern of clinical manifestations, ranging from no obvious clinical symptoms (9; present article), to prevalent neurologic signs (8–13), to neurologic signs and renal involvement (which may vary from mild urinary abnormalities to severe renal dysfunction requiring dialysis) (20–25). Such heterogeneity can occur even within individuals who carry the same ADAMTS13 genotype as exemplified by the family that we report. In this family, three siblings presented with complete ADAMTS13 deficiency. One had no sign of thrombotic microangiopathy. Another manifested pure neurologic symptoms and responded well to plasma therapy. The third had very severe renal failure and responded poorly to plasma. We hypothesized that modifier genes caused more severe disease and the renal phenotype in the last subject. We focused on genes encoding for the complement regulatory proteins factor H and MCP (34,35), because mutations in those genes have been associated with 30 to 40% familial cases of HUS and result in localized manifestations in the kidneys (31,32,36–39).

The two affected sisters had different genotypes (Figure 1B) on the area of chromosome 1q32, where factor H and MCP genes are mapped. The MCP gene was normal. However, a new heterozygous G2742T mutation in exon 18 (Figure 2B) was found in the factor H gene in patient F45, who developed chronic renal failure, but not in patient F48, who did not. The mutation that was not found in any of 100 unrelated healthy subjects causes a S890I change in short consensus repeat (SCR) 15 of factor H, which was reported recently as a hot spot (in addition to SCR20) for mutations in patients with HUS (40). It is tempting to speculate that in patient F45, factor H haploin-

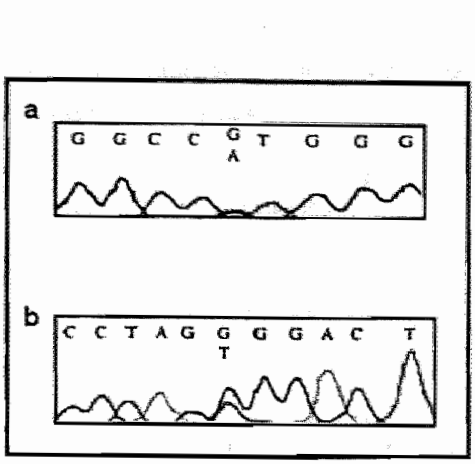


Figure 2. Mutation results. (A) Sequence of ADAMTS13 exons 3 (a) and 27 (b) showing G323A (V88M) and G3777T (G1239V) heterozygous mutations, respectively. (B) Sequence of factor H exon 18 showing a G2742T (S890I in SCR15) heterozygous mutation.

sufficiency caused uncontrolled complement activation and C3b deposition followed by microangiopathic injury in the kidney that superimposed on the systemic thrombotic microangiopathy caused by ADAMTS13 deficiency. Lower than normal serum C3 concentrations and normal C4 levels were found in patient F45, which would indicate that factor H mutation resulted in activation of the alternative pathway of complement (Figure 1B). As expected, patient F48, who does not carry the factor H mutation, had normal C3 and C4 serum concentrations.

It is interesting that the S890I factor H mutation was also found in three unaffected brothers, including F50, who also carries the ADAMTS13 defect, and in an unaffected sister (Figure 1B). In addition, the two patients experienced the first episode of thrombotic microangiopathy in adulthood during pregnancy. Altogether, these data show that ADAMTS13 and factor H gene mutations predispose to thrombotic microangiopathy and organ dysfunction and that triggers including pregnancy and viral or bacterial infections seem to play a relevant role in the full manifestation of the disease.

These results may not explain all cases of renal involvement in patients with TTP and ADAMTS13 deficiency, and additional studies, including a higher number of patients screened for factor H and for other HUS-associated genes, such as MCP and factor I, are required. However, this data disclose for the first time the genetic and phenotypic complexity of TTP and might provide a genetic explanation for cases of clinical syndrome overlapping with HUS.

Acknowledgments

This work was partially supported by grants from Comitato 30 ore per la vita, from Telethon (grants GGP02161 and GGP02162) and from Foundation for Children with Atypical HUS along with The Nando Peretti Foundation. S. Brioschi is a recipient of a fellowship from Fondazione ARMR through a contribution by The Lions Club S. Alessandro Bergamo. S. Bucchioni is a recipient of a fellowship from Fondazione ARMR through a contribution by Anna Vi Gioielleria Bergamo.

We thank Paola Bettinaglio and Francesca Porrati for complement product measurement and Gaia Pianetti for manuscript editing. We are grateful to Edoardo Rossi, Milan, for referring the family to the registry.

References

1. Moake JL: Thrombotic microangiopathies. *N Engl J Med* 347: 589–600, 2002
2. Furlan M, Robles R, Solenthaler M, Wassmer M, Sandoz P, Lammle B: Deficient activity of von Willebrand factor-cleaving protease in chronic relapsing thrombotic thrombocytopenic purpura. *Blood* 89: 3097–3103, 1997
3. Furlan M, Robles R, Galbusera M, Remuzzi G, Kyrle PA, Brenner B, Krause M, Scharrer I, Aumann V, Mittler U, Solenthaler M, Lammle B: von Willebrand factor-cleaving protease in thrombotic thrombocytopenic purpura and the hemolytic-uremic syndrome. *N Engl J Med* 339: 1578–1584, 1998
4. Tsai HM, Lian EC: Antibodies to von Willebrand factor-cleaving protease in acute thrombotic thrombocytopenic purpura. *N Engl J Med* 339: 1585–1594, 1998
5. Zheng X, Chung D, Takayama TK, Majerus EM, Sadler JE, Fujikawa K: Structure of von Willebrand factor-cleaving protease (ADAMTS13), a metalloprotease involved in thrombotic thrombocytopenic purpura. *J Biol Chem* 276: 41059–41063, 2001
6. Plaimauer B, Zimmermann K, Volkel D, Antoine G, Kerschbaumer R, Jenab P, Furlan M, Gerritsen H, Lammle B, Schwarz HP, Scheiflinger F: Cloning, expression, and functional characterization of the von Willebrand factor-cleaving protease (ADAMTS13). *Blood* 100: 3626–3632, 2002
7. Dong JF, Moake JL, Nolasco L, Bernardo A, Arceneaux W, Shrimpton CN, Schade AJ, McIntire LV, Fujikawa K, Lopez JA: ADAMTS-13 rapidly cleaves newly secreted ultralarge von Willebrand factor multimers on the endothelial surface under flowing conditions. *Blood* 100: 4033–4039, 2002
8. Levy GG, Nichols WC, Lian EC, Foroud T, McClintick JN, McGee BM, Yang AY, Siemieniak DR, Stark KR, Gruppo R, Sarode R, Shurin SB, Chandrasekaran V, Stabler SP, Sabio H, Bouhassira EE, Upshaw JD Jr, Ginsburg D, Tsai HM: Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. *Nature* 413: 488–494, 2001
9. Kokame K, Matsumoto M, Soejima K, Yagi H, Ishizashi H, Funato M, Tamai H, Konno M, Kamide K, Kawano Y, Miyata T, Fujimura Y: Mutations and common polymor-

phisms in ADAMTS13 gene responsible for von Willebrand factor-cleaving protease activity. *Proc Natl Acad Sci U S A* 99: 11902–11907, 2002
10. Schneppenheim R, Budde U, Oyen F, Angerhaus D, Aumann V, Drewke E, Hassenpflug W, Haberle J, Kentouche K, Kohne E, Kurnik K, Mueller-Wiefel D, Obser T, Santer R, Sykora KW: von Willebrand factor cleaving protease and ADAMTS13 mutations in childhood TTP. *Blood* 101: 1845–1850, 2003
11. Savasan S, Lee SK, Ginsburg D, Tsai HM: ADAMTS13 gene mutation in congenital thrombotic thrombocytopenic purpura with previously reported normal VWF cleaving protease activity. *Blood* 101: 4449–4451, 2003
12. Pimanda JE, Maekawa A, Wind T, Paxton J, Chesterman CN, Hogg PJ: Congenital thrombotic thrombocytopenic purpura in association with a mutation in the second CUB domain of ADAMTS13. *Blood* 103: 627–629, 2004
13. Matsumoto M, Kokame K, Soejima K, Miura M, Hayashi S, Fujii Y, Iwai A, Ito E, Tsuji Y, Takeda-Shitaka M, Iwadate M, Umeyama H, Yagi H, Ishizashi H, Banno F, Nakagaki T, Miyata T, Fujimura Y: Molecular characterization of ADAMTS13 gene mutations in Japanese patients with Upshaw-Schulman syndrome. *Blood* 103: 1305–1310, 2004
14. Ashida A, Nakamura H, Yoden A, Tamai H, Ishizashi H, Yagi H, Matsumoto M, Fujimura Y: Successful treatment of a young infant who developed high-titer inhibitors against VWF-cleaving protease (ADAMTS-13): Important discrimination from Upshaw-Schulman syndrome. *Am J Hematol* 71: 318–322, 2002
15. Scheiflinger F, Knobl P, Trattner B, Plaimauer B, Mohr G, Dockal M, Dorner F, Rieger M: Nonneutralizing IgM and IgG antibodies to von Willebrand factor-cleaving protease (ADAMTS-13) in a patient with thrombotic thrombocytopenic purpura. *Blood* 102: 3241–3243, 2003
16. Varadi K, Schreiner J, Plaimauer B, Rieger M, Scheiflinger F, Knobl P, Turecek PL, Schwarz HP: ADAMTS13 autoantibody detection by quantitative immunoblotting. *Blood* 102: 1932–1933, 2003
17. Klaus C, Plaimauer B, Studt JD, Dorner F, Lammle B, Mannucci PM, Scheiflinger F: Epitope mapping of ADAMTS13 autoantibodies in acquired thrombotic thrombocytopenic purpura. *Blood* 103: 4514–4519, 2004
18. Zheng XL, Kaufman RM, Goodnough LT, Sadler JE: Effect of plasma exchange on plasma ADAMTS13 metalloprotease activity, inhibitor level, and clinical outcome in patients with idiopathic and nonidiopathic thrombotic thrombocytopenic purpura. *Blood* 103: 4043–4049, 2004
19. Studt JD, Hovinga JA, Radonic R, Gasparovic V, Ivanovic D, Merkler M, Wirthmueller U, Dahinden C, Furlan M, Lammle B: Familial acquired thrombotic thrombocytopenic purpura: ADAMTS13 inhibitory autoantibodies in identical twins. *Blood* 103: 4195–4197, 2004
20. Remuzzi G, Galbusera M, Noris M, Canciani MT, Daina E, Bresin E, Contaretti S, Caprioli J, Gamba S, Ruggenenti P, Perico N, Mannucci PM: von Willebrand factor cleaving protease (ADAMTS13) is deficient in recurrent and familial thrombotic thrombocytopenic purpura and hemolytic uremic syndrome. *Blood* 100: 778–785, 2002
21. Licht C, Stapenhorst L, Simon T, Budde U, Schneppenheim R, Hoppe B: Two novel ADAMTS13 gene mutations in thrombotic thrombocytopenic purpura/hemolytic-uremic syndrome (TTP/HUS). *Kidney Int* 66: 955–958, 2004
22. Fujimura Y: Is Upshaw-Schulman syndrome congenital thrombotic thrombocytopenic purpura or hemolytic-uremic syndrome? Yes to both. *J Thromb Haemost* 1: 2457–2458, 2003
23. Assink K, Schiphorst R, Allford S, Karpman D, Etzioni A, Brichard B, van de Kar N, Monnens L, van den Heuvel L: Mutation analysis and clinical implications of von Willebrand factor-cleaving protease deficiency. *Kidney Int* 63: 1995–1999, 2003
24. Veyradier A, Obert B, Haddad E, Cloarec S, Nivet H, Foulard M, Lesure F, Delattre P, Lakhdari M, Meyer D, Girma JP, Loirat C: Severe deficiency of the specific von Willebrand factor-cleaving protease (ADAMTS 13) activity in a subgroup of children with atypical hemolytic uremic syndrome. *J Pediatr* 142: 310–317, 2003
25. Pham PT, Danovitch GM, Wilkinson AH, Gritsch HA, Pham PC, Eric TM, Kendrick E, Charles LR, Tsai HM: Inhibitors of ADAMTS13: A potential factor in the cause of thrombotic microangiopathy in a renal allograft recipient. *Transplantation* 74: 1077–1080, 2002
26. Ruggenenti P, Noris M, Remuzzi G: Thrombotic microangiopathy, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura. *Kidney Int* 60: 831–846, 2001
27. Tsai HM: Is severe deficiency of ADAMTS-13 specific for thrombotic thrombocytopenic purpura? Yes. *J Thromb Haemost* 1: 625–631, 2003
28. Remuzzi G: Is ADAMTS-13 deficiency specific for thrombotic thrombocytopenic purpura? No. *J Thromb Haemost* 1: 632–634, 2003
29. Rieger M, Ferrari S, Herzog A, Konetschny C, Dockal M, Plaimauer B, Scheiflinger F: Quantification of ADAMTS13 antigen levels in healthy donors and patients with thrombotic microangiopathies by a newly developed sandwich ELISA. *Blood* 104: 72b, 2004
30. Noris M, Ruggenenti P, Perna A, Orisio S, Caprioli J, Skerka C, Vasile B, Zipfel PF, Remuzzi G: Hypocomplementemia discloses genetic predisposition to hemolytic uremic syndrome and thrombotic thrombocytopenic purpura: Role of factor H abnormalities. Italian Registry of Familial and Recurrent Hemolytic Uremic Syndrome/Thrombotic Thrombocytopenic Purpura. *J Am Soc Nephrol* 10: 281–293, 1999
31. Caprioli J, Bettinaglio P, Zipfel PF, Amadei B, Daina E, Gamba S, Skerka C, Marziliano N, Remuzzi G, Noris M: The molecular basis of familial hemolytic uremic syndrome: Mutation analysis of factor H gene reveals a hot spot in short consensus repeat 20. *J Am Soc Nephrol* 12: 297–307, 2001
32. Noris M, Brioschi S, Caprioli J, Todeschini M, Bresin E, Porrati F, Gamba S, Remuzzi G: Familial haemolytic uraemic syndrome and an MCP mutation. *Lancet* 362: 1542–1547, 2003
33. Kokame K, Miyata T: Genetic defects leading to hereditary thrombotic thrombocytopenic purpura. *Semin Hematol* 41: 34–40, 2004
34. Walport MJ: Complement. Second of two parts. *N Engl J Med* 344: 1140–1144, 2001
35. Walport MJ: Complement. First of two parts. *N Engl J Med* 344: 1058–1066, 2001
36. Richards A, Kemp EJ, Liszewski MK, Goodship JA, Lampe AK, Decorte R, Muslumanoglu MH, Kavukcu S, Filler G, Pirson Y, Wen LS, Atkinson JP, Goodship TH: Mutations in

human complement regulator, membrane cofactor protein (CD46), predispose to development of familial hemolytic uremic syndrome. *Proc Natl Acad Sci U S A* 100: 12966–12971, 2003

37. Perez-Caballero D, Gonzalez-Rubio C, Gallardo ME, Vera M, Lopez-Trascasa M, Rodriguez de Cordoba S, Sanchez-Corral P: Clustering of missense mutations in the C-terminal region of factor H in atypical hemolytic uremic syndrome. *Am J Hum Genet* 68: 478–484, 2001
38. Caprioli J, Castelletti F, Bucchioni S, Bettinaglio P, Bresin E, Pianetti G, Gamba S, Brioschi S, Daina E, Remuzzi G, Noris M: Complement factor H mutations and gene polymorphisms in haemolytic uraemic syndrome: The C-257T, the A2089G and the G2881T polymorphisms are strongly associated with the disease. *Hum Mol Genet* 12: 3385–3395, 2003
39. Richards A, Buddles MR, Donne RL, Kaplan BS, Kirk E, Venning MC, Tielemans CL, Goodship JA, Goodship TH: Factor H mutations in hemolytic uremic syndrome cluster in exons 18–20, a domain important for host cell recognition. *Am J Hum Genet* 68: 485–490, 2001
40. Dragon-Durey MA, Fremeaux-Bacchi V, Loirat C, Blouin J, Niaudet P, Deschenes G, Coppo P, Herman Fridman W, Weiss L: Heterozygous and homozygous factor h deficiencies associated with hemolytic uremic syndrome or membranoproliferative glomerulonephritis: Report and genetic analysis of 16 cases. *J Am Soc Nephrol* 15: 787–795, 2004

CHAPTER 6

Familial haemolytic uraemic syndrome and an MCP mutation

M. Noris, S. Brioschi, J. Caprioli , M. Todeschini, E. Bresin,

F. Porrati, S. Gamba and G. Remuzzi, published in

The Lancet 2003;362:1542-1547

Mechanisms of disease

Familial haemolytic uraemic syndrome and an *MCP* mutation

*Marina Noris, Simona Brioschi, Jessica Caprioli, Marta Todeschini, Elena Bresin, Francesca Porrati, Sara Gamba, Giuseppe Remuzzi for the International Registry of Recurrent and Familial HUS/TTP**

Summary

Background Mutations in factor H (*HF1*) have been reported in a consistent number of diarrhoea-negative, non-Shiga toxin-associated cases of haemolytic uraemic syndrome (D⁻HUS). However, most patients with D⁻HUS have no *HF1* mutations, despite decreased serum concentrations of C3. Our aim, therefore, was to assess whether genetic abnormalities in other complement regulatory proteins are involved.

Methods We screened genes that encode the complement regulatory proteins—ie, factor H related 5, complement receptor 1, and membrane cofactor protein (MCP)—by PCR-single-strand conformation polymorphism (PCR-SSCP) and by direct sequencing, in 25 consecutive patients with D⁻HUS, an abnormal complement profile, and no *HF1* mutation, from our International Registry of Recurrent and Familial HUS/TTP (HUS/thrombotic thrombocytopenic purpura).

Findings We identified a heterozygous mutation in *MCP*, a surface-bound complement regulator, in two patients with a familial history of HUS. The mutation causes a change in three aminoacids at position 233–35 and insertion of a premature stop-codon, which results in loss of the transmembrane domain of the protein and severely reduced cell-surface expression of MCP.

Interpretation Results of previous studies on *HF1* indicate an association between HF1 deficiency and D⁻HUS. Our findings of an *MCP* mutation in two related patients suggest that impaired regulation of complement activation might be a factor in the pathogenesis of genetic forms of HUS. *MCP* could be a second putative candidate gene for D⁻HUS. The protein is highly expressed in the kidney and plays a major part in regulation of glomerular C3 activation. We propose, therefore, that reduced expression of *MCP* in response to complement-activating stimuli could prevent restriction of complement deposition on glomerular endothelial cells, leading to microvascular cell damage and tissue injury.

Lancet 2003; **362:** 1542–47
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*Members listed at end of paper
Mario Negri Institute for Pharmacological Research, Clinical Research Center for Rare Diseases, Aldo e Cele Daccò, Villa Camozzi-Ranica, Bergamo, Italy (M Noris Chem Pharm D, S Brioschi Biol Sci D, J Caprioli Biol Sci D, M Todeschini, E Bresin MD, F Porrati Biol Sci D, S Gamba RN, Prof G Remuzzi MD); **Department of Nephrology and Dialysis, Azienda Ospedaliera, Ospedali Riuniti di Bergamo, Italy** (Prof G Remuzzi)

Correspondence to: Dr Marina Noris, Mario Negri Institute for Pharmacological Research, Clinical Research Center for Rare Diseases "Aldo e Cele Daccò" 24020, Ranica (BG), Italy (e-mail: noris@marionegri.it)

Introduction

Haemolytic uraemic syndrome (HUS) is a rare disease of microangiopathic haemolysis, thrombocytopenia, and renal failure.[1,2] The most common form of HUS in children, with predominant renal failure, is associated with infection by *Escherichia coli*, which produce a powerful Shiga-like toxin.[1] This form of the disease (D⁺HUS) usually presents with a diarrhoea prodrome and has an excellent prognosis in most cases.[3] By contrast, non-Shiga toxin-associated and diarrhoea-negative forms of HUS (D⁻HUS) have a much poorer outcome (often end-stage renal failure or death[4–6]), with patients prone to relapse. There is sometimes a clustering of affected individuals within families, suggesting a genetic predisposition to the disease. Both autosomal dominant and autosomal recessive forms of inheritance have been noted, with precipitating events such as pregnancy, virus-like disease, or sepsis reported in some instances.[7–11] Low serum concentrations of the third component of complement (C3) have been identified in patients with D⁻HUS.[12,13] Among such patients, a subgroup—between 13% and 30%—carry mutations in the gene encoding for factor H (*HF1*), a plasma protein that inhibits the activation of the alternative pathway of complement.[14–19] However, two thirds of patients with D⁻HUS have no *HF1* mutations, despite decreased C3 concentrations,[1,18,19] indicating a role for genetic abnormalities in other complement regulatory proteins.

Methods

Participants

Between, 1996, and May, 2003, we enrolled consecutive patients with familial, recurrent, or sporadic D⁻HUS with no *HF1* mutations but an abnormal serum complement profile (defined as C3 serum concentrations <0·83 g/L[18] or a plasma C3d/serum C3 ratio >0·015) through the International Registry of Recurrent and Familial HUS/TTP (HUS/thrombotic thrombocytopenic purpura), a network of 60 Haematology and Nephrology Units established under the coordination of the Clinical Research Centre for Rare Diseases "Aldo e Cele Daccò". We also recruited healthy blood donors as controls. For protein expression studies in peripheral blood mononuclear cells (PBMC), healthy female controls and uraemic female controls on chronic haemodialysis for causes other than HUS, were recruited.

All participants received detailed information on the purposes and design of the study and provided informed written consent, according to the guidelines of the Declaration of Helsinki. The protocol was approved by the institutional review board of the "Mario Negri" Institute for Pharmacological Research.

Procedures

With respect to complement profile assessment, we quantified serum C3 and C4 concentrations by kinetic nephelometry, and ascertained serum concentrations of

GLOSSARY

CLASSICAL AND ALTERNATIVE COMPLEMENT PATHWAYS
Complement is part of the innate immune system and underlies the main effector mechanism of antibody-mediated immunity. The classical pathway is initiated by the binding of C1 complex to antibodies bound to an antigen on the surface of a bacterial cell. The alternative pathway is initiated by the covalent binding of a small amount of C3b to hydroxyl groups on cell-surface carbohydrates and proteins, and is activated by the low-grade cleavage of C3 in plasma. The two pathways lead to the formation of specific C3 and C5 convertases, converge in the formation of the membrane attack complex (MAC), and end with cell lysis.

SINGLE-STRANDED CONFORMATIONAL POLYMORPHISM (SSCP) ANALYSIS
A method for distinguishing between DNA fragments with different sequences (polymorphisms) amplified from the same genomic region based on differences in the mobility of the single-stranded DNA during polyacrylamide gel electrophoresis.

factor H, factor I, and factor B by radial immunodiffusion (RID; The Binding Site, Birmingham, UK).[12] C3d was assessed on plasma collected on EDTA by RID (The Binding Site).

We measured plasma ADAMTS13 activity as previously described,[20] using the collagen binding assay. The presence of ADAMTS13 inhibitory antibodies was assayed by testing ADAMTS13 activity in mixtures of plasma taken from patients and from a plasma pool at different dilutions after 30 min incubation at 37°C.[20]

To identify the causative gene(s) of HUS in patients with no *HF1* mutations, we looked at abnormalities in the complement regulatory proteins—factor H related 5 (FHR5),[21] complement receptor 1 (CR1),[22] and membrane cofactor protein (MCP).[23] We extracted genomic DNA from peripheral blood, according to standard protocols (Nucleon BACC2 kit, Amersham, UK). The coding sequences of *HF1*, *FHR5*, *CR1*, and *MCP* were screened by PCR-SINGLE STRANDED CONFORMATIONAL POLYMORPHISM ANALYSIS (PCR-SSCP), using primers designed on published genomic sequences (NT-004671, NT-021877).[19,20,24] A list of primers used for *MCP* gene analysis is reported in table 1. We did PCR reactions in a 20 μL volume, containing 100 ng DNA, 15 pmol of each primer, 16 nmol deoxynucleoside triphosphates (dNTP), 2·25 mmol/L magnesium chloride, 1 U Taq polymerase (Taq Gold, PE Applied Biosystems, Foster City, CA, USA), and PCR buffer. 10 min denaturation at 94°C was followed by 35 PCR cycles (94°C for 45 s, 55·5°C for 30 s, and 72°C for 45 s) and by 10 min extension at 72°C. We mixed samples with 20 μL of loading buffer, denatured them at 65°C for 10 min, and electrophoresed onto non-denaturing 6% (62/1 acryl/bis) acrylamide gel in TAE buffer (pH 6·8) at 35 Watt for 3–5 h at 4°C. We visualised gels by silver staining. Aberrant bands were sequenced.

We did expression studies in PBMC, which we separated by density gradient centrifugation with Ficoll-Paque, according to standard procedure. PBMC were incubated with a fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibody against human MCP (20 μL/10^6 PBMC, BD Biosciences Pharmingen, San Diego, CA, USA) or with FITC-mouse IgG (isotype control), and analysed by FACSort (BD Biosciences, Mountain View, CA, USA).

Role of the funding source
The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

We enrolled 25 patients with familial (n=12), recurrent (n=6), or sporadic (n=7) D⁺HUS, all of whom were white, had no *HF1* mutations, and had an abnormal serum complement profile. We also enrolled 100 healthy blood donors and, for protein expression studies in PBMC, six healthy female controls and three uraemic female controls.

Analysis of *FHR5*, indicated a heterozygous 343C→T polymorphism leading to a L66F change in short consensus repeat (SCR) 1 in two patients and in one healthy control, a heterozygous 1160G→A polymorphism leading to a R338H change in SCR6 in four patients and one healthy control, and a heterozygous 1634T→G polymorphism causing a M496R change in one patient and one control.

With respect to the membrane-bound regulatory protein CR1, we identified no mutations in the patients, and the distribution of known *CR1* polymorphisms was comparable in patients and controls.[24] In particular, quantitative expression of CR1 on cell surface is regulated by a genetic element that is linked to the site of a HindIII restriction fragment length polymorphism of the *CR1* gene that determines either a high (H) or a low (L) expression allele.[25] Among the 11 polymorphisms described in the *CR1* coding sequence, the 5507C→G (P1827R) in exon 33 is in strict linkage disequilibrium with the HindIII polymorphism: specifically, the C variant is linked to the H allele, and the G variant to the L allele.[26] The distribution of 5507C→G genotypes was similar in HUS patients (CC=55%, CG=40%, GG=5%) and in controls (CC=56%, CG=37%, GG=7%), thus excluding an association between *CR1* L allele and D⁺HUS.

Exon	Function	Primer	
		Sense	Antisense
1	Signal peptide	5'-CTGGATGCTTTGTGAGTTGGG-3'	5'-TCTTGCCCGACTGAGGAGAG-3'
2	SCR1	5'-ACTTCATCTTCATGTTCCTATTCTCTTATC-3'	5'-ACCCCAAAATGTATGCAAATCTCT-3'
3	SCR2A	5'-CAGATCTGTTTTATAACTGGATTGAAA-3'	5'-GAAGAGAAGCAAAACAAAAATAAAATT-3'
4	SCR2B	5'-GTGTGTCTATTAATTGCTATACAAAACAGT-3'	5'-AGAAACCTCTTTGGGATCTTTGTTA-3'
5	SCR3	5'-TGTCTTAATCTTTTACATTTCCTTTCCTCT-3'	5'-CACATACACCTGCTTTGTTTATCTGT-3'
6	SCR4	5'-CTTGTCTCTGTTCACACTGGAAATTACT-3'	5'-CAGCAACAACAATAACAAAACCAAGA-3'
7, 8	STP A, B	5'-CCCAAGTGGTTGATCTTCTAACATT-3'	5'-ATAAGTGAACATCACCAGAAATTTGAA-3'
9	STP C	5'-TTGATAAGGCCCTGGTGAATTT-3'	5'-CCTGCACGCTGTGCACA-3'
10	Unknown	5'-AAAATCACCCTATGAGTTTAAAGGATTT-3'	5'-CCTACACGTTTCTACACATACTACCACTTA-3'
11	TM	5'-GGAGATCCATGTGTTCAACATCTT-3'	5'-AATGCATGTCTTCACAATAATTTTTG-3'
12	TM	5'-CAGAATTATATGTCATTTGTTTCCTGG-3'	5'-AAGGACCAAGAAGTTAAAAGAAACATG-3'
13	CT	5'-TCGTTTCTTTTTGGTTTGAAGTCA-3'	5'-GCAAACCTTTCTCTCATCTCTCCT- 3'
14	CT	5'-GGCTTCTGGAATTTAATTTCTGTACTTAA-3'	5'-GTCAAAGATGAACTGGCAAACC- 3'

STP=serine-threonine-proline rich domain. TM=trans-membrane domain. CT=cytoplasmic tail.

Table 1: **Primers used for *MCP* screening**

However, a mutation in *MCP* was noted in two of 25 D⁺HUS patients—in a 21-year-old woman with a history of recurrent HUS (identified as proband in the report) and in her affected brother (table 2). The results obtained for these two individuals and their parents, form the basis of this report.

Disease onset in the proband was at age 16 months, when she developed a fever, haemolytic anaemia, and thrombocytopenia. At that time, renal function was normal. Thereafter, the patient had six recurrences of thrombotic microangiopathy, all associated with deteriorating renal function. Treatment consisted of

Parameter (normal range)	Proband	Brother
Platelets (150–400×10⁹/L)	178	247
Lactate dehydrogenase (230–460 U/L)	375	304
C3 (0·8–1·8 g/L)	0·5	1·3
C4 (0·2–0·5 g/L)	0·3	0·3
C3d (3·03–18·23 mg/L)	14·8	49·8
C3d/C3 (0·005–0·015)	0·031	0·039
Factor H (350–750 mg/L)	589·5	908
Factor B (191–382 mg/L)	210·5	262·7
Factor I (28–58 mg/L)	38·3	41·5
ADAMTS13 activity (50–150%)	68	116

Table 2: **Biochemical data for proband and her brother**

plasma exchanges and infusions, steroids, and blood transfusions, which led to complete recovery of blood abnormalities and renal function. The last episode of disease recurrence, at age 20 years, was characterised by anaemia, thrombocytopenia, and severe impairment of renal function. Transient improvement was achieved by treatment with plasma exchanges and methylprednisolone, but this response was not sustained and, despite maintenance treatment with plasma exchanges, renal function progressively deteriorated and the patient was started on a chronic haemodialysis programme. A renal biopsy showed irreversible changes of chronic nephropathy with typical features of HUS, including diffuse narrowing/occlusion of vascular vessels and severe glomerular ischaemia. The proband's brother had two episodes of HUS at age 9 years. Both episodes were characterised by severe haemolytic anaemia and acute renal insufficiency and resolved without plasma treatment, with no renal sequelae. He was referred to our registry at age 16 years. The proband and her brother had no signs of micro-angiopathic haemolysis (table 2) at the time of our examination. At that time, the proband was aged 21 years and was on chronic haemodialysis, whereas her brother had a normal renal function. Both parents are healthy with no history of renal *disease (figure 1)*.

We noted reduced serum concentrations of C3 and a higher than normal C3d/C3 ratio in the proband, whereas C4 concentrations were within the normal range, which is consistent with a selective chronic activation of the alternative pathway of complement (table 2). C3 and C4 concentrations were normal in the proband's brother, however C3d concentration and the C3d/C3 ratio were higher than normal (table 2). In the parents, concentrations of C3 (father: 1·1 g/L; mother: 0·9 g/L) and C4 (father: 0·3 g/L; mother: 0·3 g/L) were within the normal range. Factor H serum concentrations were normal in the proband and in her mother (619 mg/L), but were higher than normal range in the father (938 mg/L) and in the brother (table 2). Factor B and factor I concentrations were within the normal range (table 2).

We also measured the activity of ADAMTS13, a plasma protease that cleaves von Willebrand Factor multimers soon after their release by endothelial cells.[20] This measurement was done since deficiency of ADAMTS13 activity has been reported in patients with TTP, a thrombotic microangiopathy that shares many features with HUS, but also in some patients with HUS.[20] ADAMTS13 activity was normal in all family members (father: 106%; mother: 76%; brother: table 2), though in the proband it fell in the lower limit of normal range (table 2).

The proband and her affected brother were heterozygous for the 1160G→A polymorphism in *FHR5* and were homozygous for the C variant of the polymorphism 5507C→G in *CR1*, associated with a high expression allele (H).[26]

SSCP analysis of the *MCP* gene indicated an anomalous pattern in exon 6 (figure 1). By sequencing we found a heterozygous 2-bp deletion, causing a change in three aminoacids at position 233–35 and insertion of a premature stop-codon at position 236, which resulted in loss of the C-terminus of the protein (figure 1). The mutation was inherited by the proband from her father and was also carried by the affected brother, but was not found in the mother or in any of the 100 healthy controls.

We undertook expression studies in PBMC. FACS analysis of PBMC isolated from the proband and from the other *MCP* mutation carriers in the family (the father and

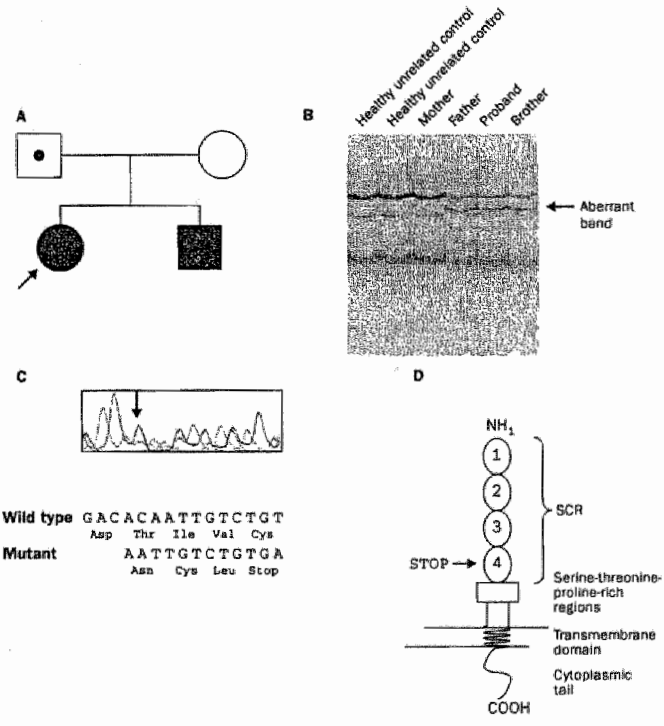

Figure 1: **Genetic studies of *MCP***

A=pedigree of family; arrow indicates proband, affected individuals in black, healthy carrier identified by black dot. B=SSCP analysis of exon 6 of *MCP*. C=sequence of exon 6 of *MCP* in proband; arrow indicates heterozygous mutation, causing 2-bp deletion (delA843-C844). D=structure and functional domains of MCP protein; arrow indicates interruption of mutant protein translation due to stop codon in SCR4, causing loss of C-terminus, including transmembrane domain.

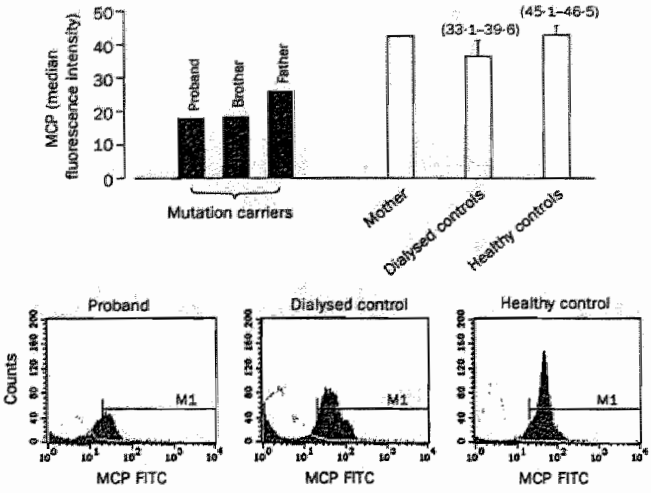

Figure 2: **Expression studies of MCP protein**

Flow cytometry analysis of MCP expression in PBMC from three mutation carriers (proband, brother and father), healthy mother, dialysed controls (n=3), and healthy controls (n=6). A: data are mean (SD); ranges in parentheses. B: histograms from proband, a representative dialysed control, and a representative healthy control.

the brother) showed around 50% reduction in *MCP* median fluorescence intensity, by comparison with PBMC from healthy controls (figure 2), indicating that the mutation affected the expression concentrations of MCP protein. By contrast the mother, who does not carry the mutation, had a normal *MCP* expression pattern (figure 2). Similar results were obtained when data were expressed as the proportion of MCP^+ cells (proband: 40%; brother: 23%; father: 50%; mother: 72% *vs* healthy controls: 86% [SD 3%]). The defect was not the consequence of uraemia, since *MCP* expression levels in PBMC from patients on chronic haemodialysis (median fluorescence intensity, figure 2; proportion MCP^+ cells: 72% [5]) were higher than those in the proband. Both the proportion of MCP^+ cells and the median fluorescence intensity (figure 2) in PBMC from the three mutation carriers lie outside the range of values of MCP^+ cells (81–90%) and MCP median fluorescence intensity recorded in PBMC from healthy controls and from individuals on haemodialysis (MCP^+ cells: 68–74%; median fluorescence intensity: figure 2).

Discussion

Our results of genetic screening in 25 consecutive D⁺HUS patients without mutations in *HF1* but presenting with abnormalities in the ALTERNATIVE but not in the CLASSICAL PATHWAY OF COMPLEMENT, led us to identify a candidate gene for D⁺HUS in addition to *HF1*. Based on the fact that these patients had signs of activation of the complement system in their blood, we focused our search for a genetic cause of the disease on genes involved in complement regulation. We identified a heterozygous mutation in the *MCP* gene in one family.

MCP is a widely expressed transmembrane glycoprotein that regulates complement activation. It serves as a cofactor for factor I to cleave C3b and C4b when they are deposited on host cells.[13] MCP has four extracellular contiguous modules important for its inhibitory activity, followed by a serine-threonine-proline rich domain, a transmembrane domain, and a cytoplasmic tail (figure 1).[23] Because the delA843-C844 mutation causes loss of the MCP C-terminus, comprising part of all of these domains, we reasoned that it might affect cell-surface expression of MCP through inhibition of insertion of the mutant protein into the plasma membrane; results of our expression studies in PBMC lend support to this notion.

Together, the findings of studies[14–19] on *HF1*, showing an association between HF1 deficiency and D⁺HUS, and our findings of an *MCP* gene mutation in two patients within one family make a strong case for impaired regulation of complement activation as a determinant factor of the disease in genetic forms of HUS. MCP is highly expressed in the kidney[27] and plays a major part in regulating glomerular C3 activation.[28] Reduced expression of MCP in the presence of stimuli that activate the complement system—eg, infection, cytotoxic drugs, antibodies, or immune complexes—might prevent restriction of complement deposition on glomerular endothelial cells, leading to microvascular cell damage and tissue injury. That the father had had no clinical manifestations of HUS despite having the same *MCP* mutation as the proband and her affected brother is consistent with an autosomal-dominant mode of transmission with reduced penetrance, as noted in *HF1* mutation carriers.[15–19] The incomplete penetrance of the disease in carriers of either *HF1*[15–19] or *MCP* mutations indicates that D⁺HUS is a complex disorder that fully manifests in the presence of environmental factors and multiple genetic modifier loci. This notion accords with our finding that, in the pedigree studied, MCP expression was higher in the father than in the proband and in her affected brother. Genetically controlled variations in serum concentrations of HF1 could also explain the incomplete penetrance of the disease in *MCP* mutation carriers. In this respect, HF1 concentrations are within normal range in the proband, but above normal in the non-affected father. Similarly, HF1 serum concentration was high in the proband's brother, who had a mild form of the disease despite the same *MCP* mutation. Our provisional interpretation is that in healthy individuals the wide range of variation in the HF1 serum concentration can be irrelevant, but in an individual with an *MCP* mutation a higher than normal amount of HF1 would be essential to compensate for the defective cofactor activity due to *MCP* haploinsufficiency.

On the basis of our data, we cannot rule out the possibility that the *MCP* mutation identified is unrelated to HUS and indicates a coincidental finding of a rare genetic variant. However, this possibility is unlikely since the mutation is present in two of 25 patients with D⁺HUS, but in none of 100 healthy controls. Additionally, MCP has a main role in complement regulation, which is defective in HUS. Genetic screening of *HF1* and *MCP* could unveil precious information for a more tailored clinical management of patients with D⁺HUS. Treatment of D⁺HUS relies on plasma exchange or infusion, however 50%[18] to 75%[19] of patients, often small babies, progress to end-stage renal disease and need replacement therapy. One of the most debated issues is whether kidney transplantation is feasible in HUS. Children with Shiga-

toxin associated HUS rarely progress to end-stage renal disease, but when they do so, transplantation often results in a good prognosis.[29] Notably, graft outcome is less favourable in children[30] and adults[31] with D⁺HUS, with recurrences occurring in around 50% of patients and graft failure developing in all of them. Among patients with D⁺HUS and *HF1* mutations, the recurrence range is 30–75%, according to different surveys.[18,32] In patients of our registry, the five individuals with D⁺HUS and *HF1* mutations who received a transplant had disease recurrence on the grafted kidney within a few weeks (range 1 week to 6 months) after surgery.[19] In view of the fact that HF1 is a plasma protein mainly of liver origin, indirect evidence suggests that a kidney transplant does not correct the *HF1* genetic defect (Noris M, unpublished). To speculate that a dysfunction in MCP, which is a membrane-bound protein highly expressed in the kidney, can be corrected by transplanting a normal kidney, is tempting. The graft, bearing wild-type MCP expressed on renal-cell surfaces, should conceivably be protected from disease recurrence.

Contributors

M Noris designed the study, interpreted the data, and drafted the report. S Brioschi did the experimental work on *MCP* and participated in data analysis and presentation. J Caprioli participated in setting up and coordinating genetic analyses and in preparing the manuscript. E Bresin and S Gamba participated in clinical data and biological sample collection, and in writing the clinical case report of the patient. M Todeschini was responsible for expression tests on *MCP*, and participated in data analysis and presentation. F Porrati was responsible for biochemical studies on complement and MCP, and participated in data analysis and presentation. G Remuzzi participated in discussion and interpretation of the data and revision of the report, and worked on the first draft of the paper. All authors contributed to the final version of the manuscript. M Noris and S Brioschi contributed equally to the paper.

Conflict of interest statement

None declared.

Members of International registry for HUS and TTP

Coordinators

G Remuzzi, P Ruggenenti (Clinical Research Center for Rare Diseases "Aldo e Cele Daccò", Ranica, Bergamo, and Division of Nephrology and Dialysis, "Ospedali Riuniti" Azienda Ospedaliera, Bergamo, Italy); M Noris, Clinical Research Center for Rare Diseases "Aldo e Cele Daccò", Ranica, Bergamo, Italy.

Investigators—Italy

M Garozzo (Division of Nephrology and Dialysis, "S. Marta e S. Venera" Hospital, Acireale, Catania); F Casucci, F Cazzato (Division of Nephrology, "Miulli" Hospital, Acquaviva delle Fonti, Bari); I M Ratsch (Pediatric Clinic, "G. Salesi" Hospital, Ancona); G Claudiani (Division of Hematology, "S. Liberatore" Hospital, Atri, Teramo); W De Simone (Division of Nephrology and Dialysis, "S. Giuseppe Moscati" Hospital, Avellino); P Dattolo (Division of Nephrology and Dialysis, "S. M. Annunziata" Hospital, Bagno a Ripoli, Firenze); R Bellantuono, T De Palo (Division of Nephrology and Dialysis, "Giovanni XXIII" Pediatric Hospital, Bari); N Larranzi (Centro Emodialisi, Bari); M Schiavoni (Assistenza Emofilici e Coagulopatici, Ospedale Policlinico Consorziale, Bari); T Barbui (Division of Hematology, "Ospedali Riuniti" Azienda Ospedaliera, Bergamo); A M Acquarolo (II Rianimazione "Spedali Civili, Azienda Ospedaliera", Brescia); O Carli, G Gregorini (Division of Nephrology and Dialysis, "Spedali Civili, Azienda Ospedaliera", Brescia); A Cao (Istituto di Clinica e Biologia dell'Età Evolutiva, Cagliari); C Setzu (Pediatric Division, "G. Brotzu" Hospital, Cagliari); A Bonadonna (Division of Nephrology and Dialysis, Presidio Ospedaliero di Camposampiero, Camposampiero, Padova); C Cascone, G Delfino (Division of Nephrology and Dialysis, "S. Giacomo" Hospital, Castelfranco Veneto, Treviso); S Li Volti (Pediatric Department, Policlinico Hospital, Catania); C Castellino (Division of Hematology, "Azienda Ospedaliera S. Croce e Carle", Cuneo); L Calacoci (Division of Immunohematology, "S. Giovanni di Dio" Hospital, Firenze); C Grimaldi (Division of Internal Medicine and Nephrology, "S. Giovanni di Dio" Hospital, Firenze); I Pela (Division of Nephrology, "A. Meyer" Hospital, Firenze); E Nesti (Division of Nephrology and Dialysis, "S. Miniato" Hospital, Firenze); M Salvadori (Division of Nephrology and Dialysis, "Careggi" Hospital, Firenze); GC Barbano, A Trivelli (Division of Nephrology, "G. Gaslini" Pediatric Institute, Genova); I Fontana (Transplant Center, "S. Martino" Hospital, Genova); S D'Ardia (Division of Immunohematology, Ivrea Hospital, Ivrea, Torino); C Marseglia (Service of Nephrology and Dialysis, "Carlo Poma" Hospital, Mantova); A Bettinelli (Pediatric Division, "S. Leopoldo Mandic" Hospital, Merate, Lecco); G Ardissino, A Edefonti (Division of Pediatric Nephrology, Dialysis and Transplant, "De Marchi" Pediatric Clinic, Milano); A Lattuada, E Rossi (Division of Hematology, "L. Sacco" Hospital, Milano); V Rossi (Division of Hematology, "Niguarda Cà Granda" Hospital, Milano); V Toschi (Transfusional Center, "San Carlo Borromeo" Hospital, Milano); E Gaiani, M Leonelli (Division of Nephrology, Dialysis and Transplant, Policlinico Hospital, Modena); D Belotti, E Pogliani (Division of Hematology and Transfusional Center, "S. Gerardo" Hospital, Monza, Milano); L Murer (Pediatric Division, Policlinico Hospital, Padova); A Indovina, R Marcenò (Division of Hematology, "V. Cervello" Hospital, Palermo); L Amico (Division of Nephrology and Dialysis, "V. Cervello" Hospital, Palermo); E Trabassi (Division of Nephrology and Dialysis, "San Massimo" Hospital, Penne, Pescara); G Agnelli (Division of Internal Medicine, University of Perugia); R Caprioli (Division of Nephrology and Dialysis, "S. Chiara" Hospital, Pisa); G Garozzo (Trasfusional Center, "M.P. Arezzo" Hospital, Ragusa); E Bresin, E Daina, S Gamba (Clinical Research Center for Rare Diseases "Aldo e Cele Daccò", Ranica, Bergamo); G Ene (Division of Nephrology, Clinical Physiology Center, "Consiglio Nazionale delle Ricerche", Reggio Calabria); C Zoccali (Division of Nephrology and Dialysis, "G. Monasterio" Hospital, Reggio Calabria); A Amendola, L Dessanti, F Mandelli, G Meloni (Department of Cellular Biotechnology and Hematology, "La Sapienza" University, Roma); L De Petris, S Rinaldi, GF Rizzoni (Division of Nephrology and Dialysis, "Bambino Gesù" Pediatric Hospital, Roma); T Cicchetti, G Putorti (Division of Nephrology and Dialysis, "N. Giannettasio" Hospital, Rossano Calabro, Cosenza); R Paolini (Medical Division, Rovigo Hospital, Rovigo); A Pinto (Division of Nephrology and Dialysis, "S.G. di Dio e Ruggi d'Aragona" Hospital, Salerno); A Del Giudice (Division of Nephrology, "Casa Sollievo delle Sofferenza" Hospital, S. Giovanni Rotondo, Foggia); PR Scalzulli (Division of Hematology, "Casa Sollievo delle Sofferenza" Hospital, S. Giovanni Rotondo, Foggia); M Sanna (Division of Medical Pathology, Sassari Hospital, Sassari); A Amore, R Coppo, L Peruzzi (Division of Nephrology and Dialysis, "Regina Margherita" Pediatric Hospital, Torino); A Khaled (Division of Nephrology, "S. Chiara" Hospital, Trento); M Pennesi (Division of Pediatric Nephrology, "Burlo Garofalo" Pediatric Institut, Trieste); O Amaruda (Division of Nephrology, "Fondazione Macchi" Hospital, Varese); L Tavecchia (Division of Hematology, "Borgo Roma" Hospital, Verona).

Investigators—abroad

J Ferraris (Division of Nephrology, "Hospital Italiano de Buenos Aires", Buenos Aires, Argentina); MG Caletti, M Adragna ("Juan P. Garrahan" Hospital de Pediatria, Buenos Aires, Argentina); R Wens (Clinique de Nephrologie-Dialyse, CHU Brugmann, Bruxelles, Belgium); T Ring (Department of Nephrology, Aaolborg Hospital, Aaolborg, Denmark); C Buehrer (Department of Neonatology, Charité Campus Virchow-Klinikum, Berlin, Germany); D Mueller (Department of Pediatric Nephrology, Charité, Berlin Germany); B Hoppe (University Children'Hospital, Cologne, Germay); D Landau (Division of Pediatric Nephrology, Soroka Medical Center, Beer-Sheba, Israel); P Ponce (Hospital "Garcia de Orta", Almada, Portugal); J Barbot, Antunes M (Division of Hematology, "Maria Pia" Hospital, Porto, Portugal); A Sharma (Royal Liverpool and Broadgreen University Hospitals, Liverpool, UK); GB Haycock (Pediatric Renal Unit, Guy's Hospital, London, UK); L Milner (Division of Nephrology, Floating Hospital for Children, Boston, MA, USA); JC Lane, CB Langman (Division of Kidney Diseases, Children's Memorial Hospital, Chicago, IL, USA); AM Simckes (Children's Mercy Hospital, Kansas City, MO, USA); J Gitomer (Department of Nephrology, Marshfield Clinic, Marshfield, WI, USA); SB Conley (Department of Nephrology, St. Christopher's Hospital for Children, Philadelphia, USA); D Milliner (Mayo Clinic, Rochester, MN, USA); V Kimonis (Department of Pediatrics, SIU School of Medicine, Springfield, IL, USA); J Listman (SUNY Upstate Medical University, Syracuse, NY, USA).

Laboratory analysis

F Gaspari (Clinical Research Center for Rare Diseases "Aldo e Cele Daccò", Ranica, Bergamo, Italy); C Ottomano, A Vernocchi (Division of Laboratory Analysis, "Ospedali Riuniti" Azienda Ospedaliera Bergamo, Italy).

Biochemical studies

P Bettinaglio, S Brioschi, S Bucchioni, J Caprioli, F Castelletti, D Cugini, G Pianetti (Clinical Research Center for Rare Diseases "Aldo e Cele Daccò", Ranica, Bergamo, Italy); C Capoferri, DM Galbusera, S Gastoldi ("M. Negri" Institute for Pharmacological Research, Bergamo, Italy); PF Zipfel (Hans Knoell Institute for Natural Products Research, Jena, Germany).

Statistical analysis
A Perna (Clinical Research Center for Rare Diseases "Aldo e Cele Daccò", Ranica, Bergamo, Italy).

Acknowledgments
This work was supported in part by grants from Comitato 30 ore per la vita and from Telethon (GPP02161) and by a grant from the Foundation for Children with Atypical HUS along with the Nando Peretti Foundation. S Brioschi is a recipient of a fellowship from Associazione Ricerche Malattie Rare through the generosity of the Lions Club Bergamo—St Alessandro. F Porrati is a recipient of a fellowship in memory of Libera Dossia Grana.

We thank Paola Bettinaglio for complement product measurement, Sara Bucchioni for DNA sequencing, Miriam Galbusera for ADAMTS13 assessment, and Erica Daina and Mauro Abbate for helpful discussion.

References

1 Ruggenenti P, Noris M, Remuzzi G. Thrombotic microangiopathy, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura. *Kidney Int* 2001; 60: 831–46.
2 Ruggenenti, P, Noris M, Remuzzi G. Thrombotic microangiopathies. In: Brady HR, Wilcox CS, eds. Therapy in nephrology and hypertension: a companion to Brenner and Rector's the kidney. Philadelphia: WB Saunders Company, 2003.
3 Kaplan BS, Meyers KE, Schulman SL. The pathogenesis and treatment of hemolytic uremic syndrome. *J Am Soc Nephrol* 1998; 9: 1126–33.
4 Fitzpatrick MM, Walters MDS, Trompeter RS. Atypical (non-diarrhea-associated) hemolytic-uremic syndrome in childhood. *J Pediatr* 1993; 122: 532–37.
5 Proesmans W. Typical and atypical hemolytic uremic syndrome. *Kidney Blood Press Res* 1996; 19: 205–08.
6 Neuhaus TJ, Calonder S, Leumann EP. Heterogeneity of atypical hemolytic uremic syndrome. *Arch Dis Child* 1997; 76: 518–21.
7 Kaplan BS, Kaplan P. Hemolytic uremic syndrome in families. New York: Marcel Dekker, 1992.
8 Berns JS, Kaplan BS, Mackow RC, Hefter LG. Inherited hemolytic uremic syndrome in adults. *Am J Kidney Dis* 1992; 19: 331–34.
9 Kirchner KA, Smith, RM, Gockerman JP, Luke RG. Hereditary thrombotic thrombocytopenic purpura: microangiopathic hemolytic anemia, thrombocytopenia, and renal insufficiency occurring in consecutive generations. *Nephron* 1982; 30: 28–30.
10 Bergstein J, Michael A Jr, Kjellstrand C, Simmons R., Najarian J. Hemolytic-uremic syndrome in adult sisters. *Transplantation* 1974; 17: 487–89.
11 Pirson Y, Lefebvre C, Amout C, de Strihou CY. Hemolytic uremic syndrome in three adult siblings: a familial study and evolution. *Clin Nephrol* 1997; 28: 250–55.
12 Noris M, Ruggenenti P, Perna A, et al, on behalf of the Italian Registry of Familial and Recurrent HUS/TTP. Hypocomplementemia discloses genetic predisposition to hemolytic uremic syndrome and thrombotic thrombocytopenic purpura: role of factor H abnormalities. *J Am Soc Nephrol* 1999; 10: 281–93.
13 Thompson RA, Winterborn MH. Hypocomplementaemia due to a genetic deficiency of β1H globulin. *Clin Exp Immunol* 1981; 46: 110–19.
14 Warwicker P, Goodship THJ, Donne RL, et al. Genetic studies into inherited and sporadic haemolytic uraemic syndrome. *Kidney Int* 1998; 53: 836–44.
15 Caprioli J, Bettinaglio P, Zipfel PF, et al. The molecular basis of familial hemolytic uremic syndrome: mutation analysis of factor H gene reveals a hot spot in short consensus repeat 20. *J Am Soc Nephrol* 2001; 12: 297–307.
16 Richard A, Buddles, MR, Donne RL, et al. Factor H mutations in hemolytic uremic syndrome cluster in exons 18–20, a domain important for host cell recognition. *Am J Hum Genet* 2001; 68: 485–90.
17 Perez-Caballero D, Gonzalez-Rubio C, Gallardo ME, et al. Clustering of missense mutations in the C-terminal region of factor H in atypical hemolytic uremic syndrome. *Am J Hum Genet* 2001; 68: 478–84.
18 Neumann HP, Slazmann M, Bohnert-Iwan B, et al. Haemolytic uraemic syndrome and mutations of the factor H gene: a registry-based study of German speaking countries. *J Med Genet* 2003; 40: 676–81.
19 Caprioli J, Castelletti F, Bucchioni S, et al. Complement factor H gene mutations and polymorphisms in haemolytic uraemic syndrome: the C-257T, the A2089G and the G2881T polymorphisms are strongly associated with the disease. *Hum Mol Genet* (in press).
20 Remuzzi G, Galbusera M, Noris M, et al. von Willebrand factor cleaving protease (ADAMTS13) is deficient in recurrent and familial thrombotic thrombocytopenic purpura and hemolytic uremic syndrome. *Blood* 2002; 100: 778–85.
21 McRae JL, Cowan PJ, Power DA, et al. Human factor H-related protein 5 (FHR-5): a new complement-associated protein. *J Biol Chem* 2001; 276: 6747–54.
22 Krych-Goldberg M, Atkinson JP. Structure-function relationships of complement receptor type 1. *Immunol Rev* 2001; 180: 112–22.
23 Liszewski MK, Leung M, Cui W, et al. Dissecting sites important for complement regulatory activity in Membrane cofactor Protein (MCP; CD46). *J Biol Chem* 2000; 48: 37692–701.
24 Moulds JM, Zimmerman PA, Doumbo OK, et al. Molecular identification of Knops blood group polymorphisms found in long homologous region D of complement receptor 1. *Blood* 2001; 97: 2879–85.
25 Wilson JG, Murphy EE, Wong WW, Klickstein LB, Weis JH, Fearon DT. Identification of a restriction length polymorphism by a CR1 cDNA that correlates with the number of CR1 on erythrocytes. *J Exp Med* 1986; 164: 50–59.
26 Xiang L, Rundles JR, Hamilton DR, Wilson JG. Quantitative alleles of CR1: coding sequence analysis and comparison of haplotypes in two ethnic groups. *J Immunol* 1999; 163: 4939–45.
27 Nangaku M. Complement regulatory proteins in glomerular diseases. *Kidney Int* 1998; 54: 1419–28.
28 Nakanishi I, Moutabarrik A, Hara T, et al. Identification and characterization of membrane cofactor protein (CD46) in the human kidneys. *Eur J Immunol* 1994; 24: 1529–35.
29 Ferraris JR, Ramirez JA, Ruiz S, et al. Shiga toxin-associated hemolytic uremic syndrome: absence of recurrence after renal transplantation. *Pediatr Nephrol* 2002; 17: 809–14.
30 Miller RB, Burke BA, Schmidt WJ, et al. Recurrence of haemolytic-uraemic syndrome in renal transplants: a single-centre report. *Nephrol Dial Transplant* 2002; 17: 809–14.
31 Artz MA, Steenbergen EJ, Hoitsma AJ, Monnens LAH, Wetzels JFM. Renal transplantation in patients with hemolytic uremic syndrome: high rate of recurrence and increased incidence of acute rejections. *Transplantation* 2003; 78: 821–26.
32 Loirat C, Niaudet P. The risk of recurrence of hemolytic uremic syndrome after renal transplantation in children. *Pediatr Nephrol*; published online Sept 17, 2003, DOI: 10.1007/s00467-003-1289-8.

CHAPTER 7

Disease of the month

Hemolytic uremic syndrome

M. Noris and G. Remuzzi , published in

Journal of the American Society of Nephrology 2005;16:1035-1050

Hemolytic Uremic Syndrome

Marina Noris* and Giuseppe Remuzzi*†

*Transplant Research Center, "Chiara Cucchi de Alessandri e Gilberto Crespi," Mario Negri Institute for Pharmacological Research; and †Department of Medicine and Transplantation, Ospedali Riuniti di Bergamo, Bergamo, Italy

J Am Soc Nephrol 16: 1035-1050, 2005. doi: 10.1681/ASN.2004100861

Hemolytic uremic syndrome (HUS) is a disease of nonimmune (Coombs negative) hemolytic anemia, low platelet count, and renal impairment (1). Anemia is severe and microangiopathic in nature, with fragmented red blood cells (schistocytes) in the peripheral smear, high serum lactate dehydrogenase (LDH), circulating free hemoglobin, and reticulocytes. Platelet count is $<60,000/mm^3$ in most cases (1).

In children, the disease is most commonly triggered by Shiga-like toxin (Stx)-producing *Escherichia coli* (Stx-*E. coli*) and manifests with diarrhea (D^+HUS), often bloody. Cases of Stx-*E. coli* HUS—approximately 25% (2)—which, however do not present with diarrhea, have also been reported (3). Acute renal failure manifests in 55 to 70% of cases (4–6); however, renal function recovers in most of them (up to 70% in various series) (1,3,6,7).

Non–Shiga toxin-associated HUS (non–Stx-HUS) comprises a heterogeneous group of patients in whom an infection by Stx-producing bacteria could be excluded as cause of the disease. It can be sporadic or familial (*i.e.*, more than one member of a family affected by the disease and exposure to Stx-*E. coli* excluded). Collectively, non–Stx-HUS forms have a poor outcome. Up to 50% of cases progress to ESRD or have irreversible brain damage, and 25% may die during the acute phase of the disease (8–10). Genetic studies have recently documented that the familial form is associated with genetic abnormalities of complement regulatory proteins, and evidence is now emerging that similar genetic alterations can predispose to sporadic cases of non–Stx-HUS as well. Major recent advances in the field of Stx-HUS and non–Stx-HUS are summarized in Table 1.

Microvascular lesion of HUS consists of vessel wall thickening with endothelial swelling and accumulation of proteins and cell debris in the subendothelial layer, creating a space between endothelial cells and the underlying basement membrane of affected microvessels (1,3). In Stx-HUS, the lesion is mainly confined to the glomerular tuft and is noted in an early phase of the disease. Examination of biopsies taken several months after the disease onset showed that most glomeruli are normal, whereas 15 to 20% eventually became sclerotic (11,12). Arterial thrombosis does occur but is uncommon and seems to be a proximal extension of the glomerular lesion (11,12).

Stx-Associated HUS

Epidemiology

In 70% of cases in North America and Western Europe, Stx-HUS is secondary to infection with the *E. coli* serotype O157:H7 (13–19). This serotype has a unique biochemical property (lack of sorbitol fermentation) as to render it readily distinguishable from other fecal *E. coli* (20). However, many other *E. coli* serotypes (O111:H8, O103:H2, O121, O145, O26, and O113 [13,16,21–23]) have been shown to cause Stx-HUS. Infection by Stx-producing *Shigella dysenteriae* serotype 1 has been commonly linked to Stx-HUS in developing countries of Asia (24) and Africa (25) but rarely in industrialized countries (26).

After exposure to Stx-*E. coli*, 38 to 61% of individuals develop hemorrhagic colitis and 3 to 9% (in sporadic infections) to 20% (in epidemic forms) progress to overt HUS (5,27). The overall incidence of Stx-HUS is estimated to be 2.1 cases per 100,000 persons/yr, with a peak incidence in children who are younger than 5 yr (6.1 per 100,000/yr), and the lowest rate in adults who are 50 to 59 yr of age (0.5 per 100,000/yr) (1). The incidence of the disease parallels the seasonal fluctuation of *E. coli* O157:H7 infections with a peak in warmer months, between June and September. In the United States, approximately 70,000 illnesses and 60 deaths have been attributed annually to Stx-HUS (28). In Argentina and Uruguay, *E. coli* infections are endemic and Stx-HUS is a common cause of acute renal failure in children (23,29,30), with an estimated incidence rate of 10.5 per 100,000/yr (31). An association between traditional extensive production of cattle with endemic HUS in Argentina has been proposed, as supported by detection of Stx-producing *E. coli* strains—mainly O8, O25, O103, O112, O113, O145, O171, and O174 serotypes—in stool samples from 39% of Argentine healthy young beef steers (31).

Stx-producing *E. coli* colonize healthy cattle intestine but also have been isolated from deer, sheep, goats, horses, dogs, birds, and flies (1,32). They are found in manure and water troughs in farms, which explains the increased risk for infection in people who live in rural areas. Humans become infected from contaminated milk, meat, and water—water-borne outbreaks have occurred as a result of drinking and swimming in unchlorinated water (21)—or from contact with infected animals, humans, or either's excreta (27,33,34) and occasionally through

Published online ahead of print. Publication date available at www.jasn.org.

Address correspondence to: Dr. Marina Noris, Transplant Research Center, "Chiara Cucchi de Alessandri e Gilberto Crespi," Villa Camozzi, Via Camozzi, 3 24020, Ranica (BG), Italy. Phone: +39-035-4535362; Fax: +39-035-4535377; E-mail: noris@marionegri.it

ISSN: 1046-6673/1604-1035

Table 1. HUS: Major advances in recent years[a]

Stx-HUS	
1994–2004	Description of the crystal structure of Stx-1 and Stx-2 (46,52)
1993–2001	Specific Stx surface receptors (globotriaosylceramide, Gb3) were identified on human endothelial cells, platelets, monocytes, erythrocytes, and polymorphonuclear cells (57–66)
1995–2003	Identification of the molecular mechanisms by which Stx promotes leukocyte adhesion to endothelial cells and induces thrombus formation (71–74)
2004	Description of the beneficial effect of angiotensin-converting enzyme inhibitors on long-term renal outcome in children with renal sequelae after severe Stx-HUS (83,84)
2002–2003	Reports on the good outcome of kidney transplantation in children with Stx-HUS (85–87)
Non–Stx-HUS	
1999	Description of high incidence of hypocomplementemia (low C3 levels) in familial forms of non-Stx-HUS (98)
1998	Linkage mapping of familial HUS on human chromosome 1q32 containing the regulator of complement activation gene cluster (110)
1998–2004	Identification of 50 mutations in factor H gene in familial and sporadic non–Stx-HUS (99–102,110,114,119–121)
2002–2004	Localization (in SCR19–20) of the domain responsible for inactivation of surface-bound C3b by factor H (115–118)
2002–2004	Demonstration that mutations found in patients with non–Stx-HUS cause loss of the capability of factor H to bind polyanions on endothelial cells and extracellular matrix and to bind C3b (117,118,123)
2003	Mutations in another complement regulatory gene, MCP, in non–Stx-HUS (126,127)
1997–2003	Description of high incidence of recurrence on the kidney graft in patients with non–Stx-HUS (1,85,86,99–101,153,154)
2003–2004	Complement inhibitors are being clinically available (163–167)

[a]HUS, hemolytic uremic syndrome; Stx, Shiga toxin; MCP, monocyte chemoattractant protein.

environmental contamination (17). Meat is contaminated at slaughter. Internalization of the microorganism during grinding renders it capable of surviving cooking (27). Fruits and vegetables may also be contaminated, including radish sprouts, lettuce, and apple cider. Unpasteurized apple juice has been implicated in several outbreaks (35). Person-to-person transmission has been reported in child care and long-term care facilities (27).

Clinical Phenotype

The disease is characterized by prodromal diarrhea followed by acute renal failure. The average interval between *E. coli* exposure and illness is 3 d (range, 1 to 8). Illness typically begins with abdominal cramps and nonbloody diarrhea; diarrhea may become hemorrhagic in 70% of cases usually within 1 or 2 d (36). Vomiting occurs in 30 to 60% of cases, and fever occurs in 30%. Leukocyte count is usually elevated, and a barium enema may demonstrate "thumb-printing," suggestive of edema and submucosal hemorrhage, especially in the region of the ascending and transverse colon. HUS is usually diagnosed 6 d after the onset of diarrhea (1). After infection, Stx-*E. coli* may be shed in the stools for several weeks after the symptoms are resolved, particularly in children <5 yr of age (1). Diagnosis rests on detection of Stx-*E. coli* in stool cultures. Serologic tests for antibodies to Stx and O157 LPS can be done in research laboratories, and tests are being developed for rapid detection of *E. coli* O157:H7 and Stx in stools. Blood diarrhea, fever, vomiting, elevated leukocyte count, extremes age, and female gender as well as the use of antimotility agen (37) have been associated with an increased risk of HUS after *coli* infection (27).

Stx-HUS is not a benign disease. Seventy-percent of patien who develop HUS require red blood cell transfusions, 50 need dialysis, and 25% have neurologic involvement, includin stroke, seizure, and coma (6,27,38). Although mortality for i fants and young children in industrialized countries decrease when dialysis became available, as well as after the introdu tion of intensive care facilities, still 3 to 5% of patients di during the acute phase of Stx-HUS (6). A recent meta-analys of 49 published studies (3476 patients, mean follow-up of 4. yr) describing long-term prognosis of patients who survived a episode of Stx-HUS reported death or permanent ESRD in 12 of patients and GFR <80 ml/min per 1.73 m^2 in 25% (38). Th severity of acute illness, particularly central nervous syste symptoms, and the need for initial dialysis were strongly ass ciated with a worse long-term prognosis (4,38). Stx-HUS that i precipitated by *S. dysenteriae* infection is almost invariably com plicated by bacteremia and septic shock, systemic intravascula coagulation, and acute cortical necrosis and renal death and ha a high mortality rate (approximately 30%) (39).

History of a Discovery

E. coli has been associated with hemorrhagic colitis and organ failure, including kidney failure. In 1927, Albert Adam first reported an epidemia of bloody diarrhea of infants caused by a special type of Bacterium *coli*. Such bacterium was biochemically unique in that fermentation properties were different from known *E. coli* strains (40). In 1947, the *E. coli* O111:B4 was found in the stools of >90% of infants with epidemic diarrhea but never in their blood (41). A filterable agent—we now know that this was likely Stx—that caused diarrhea in calves and was lethal to mice was isolated from the stools of these children. A few years later, it was found that most severe cases of O111: B4-induced epidemic diarrhea were associated with purpura, anuria, and neurologic signs. Autopsy material revealed thrombosis of capillary and precapillary arterioles in lungs, liver, brain, and kidneys, as well as glomerular tuft occlusion by fibrin thrombi (42). These early findings were taken to indicate that a toxin, possibly released by the *E. coli*, induced hemorrhagic necrosis of the gastrointestinal mucosa and—once absorbed into the blood stream—caused microvascular thrombosis of kidneys and the other organs. Several years later, in 1977, Konowalciuck *et al.* (43) noted that *E. coli* that was isolated from patients with diarrhea produced a toxin similar to the one of *S. dysenteriae type 1* (Stx) found cytopathic to Vero cells (African green monkey kidney cells). Karmali *et al.* (14) found an increased Stx activity in fecal filtrates and increased Stx-neutralizing antibody titer in sera from children who had *E-coli* O157:H7 infection an had received a diagnosis of HUS.

Shiga Toxin or Shiga Toxins?

The Stx associated with *E. coli* are designated by a number. Stx-1 is almost identical to Stx from *S. dysenteriae type 1*, differing by a single amino acid, and is 50% homologous with Stx-2 (44–46). Despite their similar sequences, Stx-1 and Stx-2 cause different degrees and types of tissue damage as documented by the higher pathogenicity of strains of *E. coli* that produce only Stx-2 than of those that produce Stx-1 alone (47–49). In a recent study in children who become infected by Stx-*E. coli*, *E. coli* strains that produced Stx-2 were most commonly associated with HUS, whereas most strains that were isolated from children who had diarrhea alone or remained asymptomatic produced only Stx-1 (50). This is also true in mice and baboons (45,51).

Both Stx-1 and Stx-2 are 70-kD AB5 holotoxins that are composed of a single A subunit of 32-kD and five 7.7-kD B subunits (52) (Figure 1). It is interesting that a new AB5 toxin that comprises a single 35-kD A subunit and a pentamer of 13-kD B subunits has been recently isolated from a highly virulent *E. coli* strain (0113:H21) that was responsible for an outbreak of HUS (53), which may represent the prototype of a new class of toxins, accounting for HUS associated with strains of *E. coli* that do not produce Stx.

After oral ingestion, Stx-*E. coli* reaches the gut and closely adheres to the epithelial cells of the gastrointestinal mucosa through a 97-kD outer membrane protein, intimin (54). Stx then are picked up by polarized gastrointestinal cells via transcellular pathways (55) and translocate into the circulation, probably

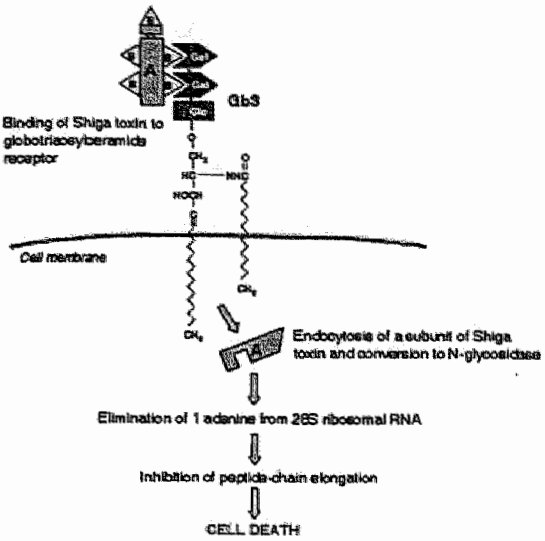

Figure 1. Binding and mechanism of action of Shiga-like toxin. The B subunits of Shiga toxin (Stx) molecules attach to galactose (gal) disaccharides of globotriaosylceramide (Gb3) receptors on the membrane of monocytes, polymorphonuclear cells, platelets, glomerular endothelial cells, and tubular epithelial cells. The toxin is internalized via retrograde transport through the Golgi complex. Then the A and B subunits dissociate, and the A subunit is translocated to the cytosol. The A subunit blocks peptide chain elongation by eliminating one adenine from the 28S ribosomal RNA.

facilitated by the transmigration of neutrophils (PMN) (56), which increase paracellular permeability. The route of transport of Stx from the intestine to the kidney has been greatly debated. *In vitro* experiments have shown that Stx can bind to human erythrocytes (57), platelets (58), and activated monocytes (59). However, more recent studies have underpinned a role for PMN in Stx transfer in the blood because Stx rapidly and completely bind to PMN when incubated with human blood (60). Consistently, Stx bound to circulating PMN have been detected in the blood of patients with Stx-HUS (61). The Stx receptor on PMN has a 100-fold lower affinity than the high-affinity receptor expressed on glomerular endothelial cells. *In vitro*, in co-cultures, PMN loaded with Stx transfer the ligand to glomerular endothelial cells so that at the end of the incubation, Stx molecules were found on glomerular endothelial cells but no more on PMN (60).

Binding of Stx to target cells is dependent on B subunits and occurs via the terminal digalactose moiety of the glycolipid cell surface receptor globotriaosylceramide Gb3 (Figure 1). Stx-1 and Stx-2 bind to different epitopes on the Gb3 molecule, and they also differ in binding affinity and kinetics (62). Surface plasmon resonance analysis showed that Stx-1 easily binds to and detaches from Gb3, in contrast to Stx-2, which binds slowly but also dissociates very slowly, thus staying on the cells long

enough to be incorporated (62). The latter could explain why Stx-2 is 1000-fold more toxic than Stx-1 on human endothelial cells *in vitro* (63).

Cultured human microvascular endothelial cells are more susceptible to the toxic effects of Stx than large-vessel endothelium (64). This is consistent with data that the number of Gb3 receptors expressed on human microvascular endothelial cells is 50-fold higher than in endothelial cells from human umbilical veins (65). In human glomerular endothelial cells, Gb3 expression and Stx toxicity are further increased upon exposure to TNF-α (66), in turn released by monocytes in response to Stx binding (59). Altogether these data provide the biochemical basis for the preferential localization of microangiopathic lesions to renal vasculature in HUS in humans.

After internalization by receptor-mediated endocytosis, Stx are carried by retrograde transport through the Golgi complex to the endoplasmic reticulum, where the A and B subunits likely dissociate (Figure 1). Then the A subunit is translocated to the cytosol and nuclear envelope, where it enzymatically blocks protein synthesis (67) (Figure 1). Stx-1 and Stx-2 also induce endothelial apoptosis (68,69) possibly by inhibiting the expression of the antiapoptotic Bcl-2 family member, Mcl-1 (70).

For many years, it was assumed that the only relevant biologic activity of Stx was the block of protein synthesis and destruction of endothelial cells. Recently, however, it has been shown that treatment of endothelial cells with sublethal doses of Stx, exerting minimal influence on protein synthesis, leads to increased mRNA levels and protein expression of chemokines, such as IL-8 and monocyte chemoattractant protein-1 (MCP-1) and cell adhesion molecules, a process preceded by NF-κB activation (71). Analysis of genome-wide expression pattern of human endothelial cells stimulated with sublethal doses of Stx evidenced 25 and 24 genes upregulated by Stx-1 and Stx-2, respectively, mostly encoding for chemokines and cytokines, cell adhesion molecules, including P-selectin and ICAM-1, and transcription factors (EGR-1, NF-κB2, and NF-κBIA) (72). Chemokines and cytokines are likely involved in the chemoattrac tion and activation of neutrophils. Adhesion molecules seem t play a critical role in mediating binding of inflammatory cells t the endothelium. This is supported by adhesion experiment under flow showing that Stx-2 treatment enhanced the numbe of leukocytes that adhere and migrate across a monolayer c human endothelial cells (73). Preventing IL-8 and MCP-1 ove expression by adenovirus-mediated blocking of NF-κB inhib ited the adhesion and transmigration of leukocytes (71).

Taken together, these findings indicate that Stx, by alterin endothelial cell adhesion properties and metabolism, favor leu kocyte-dependent inflammation. The latter activates endothe lial cells that lose thromboresistance, which ultimately leads t microvascular thrombosis. Evidence for such sequence c events has been obtained in experiments of whole blood flow ing on human microvascular endothelial cells, pre-exposed t Stx-1, at high shear stress (74). Finding that in such circum stances early platelet activation and adhesion takes place, fol lowed by the formation of organized thrombi dependent o endothelial P-selectin and PECAM-1, offers a plausible patho physiologic pathway for microvascular thrombosis in HUS The above report could also be taken as a demonstration of link between bacteria and their products and arterial thrombo sis, as suggested in the accompanying commentary (75).

In vivo evidence of coagulation disturbances, *i.e.*, increase i prothrombin fragment 1 + 2, has been found (36) in childre who developed HUS upon *E. coli* O157:H7 infection. Althoug early studies suggested that fibrinolysis is augmented in Stx HUS (76), more recent work revealed the presence of higher than-normal levels of plasminogen-activator inhibitor type 1 indicating that fibrinolysis is substantially inhibited (36).

Is There Any Effective Treatment for Stx-HUS?

There is no treatment of proven value, and care during th acute phase of the illness is still merely supportive with n substantial changes as compared with the past (Table 2). Ther is no clear consensus on whether antibiotics should be admin

Table 2. Classification and treatment of different forms of HUS[a]

Disease	Causes	Treatment
Stx-HUS	Stx-producing *Escherichia coli*	Supportive
	Shigella dysenteriae type 1	Supportive, antibiotics
Non-Stx-HUS	Bacteria (*Streptococcus pneumoniae*)	Antibiotics, no plasma
sporadic	Viruses (HIV)	Plasma
	Drugs (antineoplastic, antiplatelet, immunosuppressive)	Drug withdrawal, plasma
	Pregnancy associated	Delivery, plasma
	Postpartum	Plasma
	Systemic diseases	
	lupus	Steroids, plasma
	scleroderma	BP control
	antiphospholipid syndrome	Oral anticoagulants
	Idiopathic	Plasma
	Genetic (factor H, MCP, factor I)	Plasma
familial	Genetic (factor H, MCP, factor I), plasma	Plasma

istered to treat Stx-*E. coli* infection. Wong *et al.* (77) showed that antibiotic therapy at the stage of gastrointestinal infection with Stx-*E. coli* increases—by approximately 17-fold—the risk for full-blown HUS. It was postulated that antibiotic-induced injury to the bacterial membrane might favor the acute release of large amounts of toxins. However, a recent meta-analysis on 26 reports failed to show a higher risk for HUS associated with antibiotic administration (78). Of note, in the study by Wong *et al.*, none of the patients had bacteremia. Although bacteremia is very common in Stx-HUS precipitated by *S. dysenteriae type 1* and these patients eventually progress to death unless antibiotics are started early enough (79,80), such complication is only exceptionally found in Stx-HUS sustained by *E. coli* O157:H7 infection. However, a recent report of an adult patient with *E. coli* O157:H7–induced HUS with bacteremia and urinary tract infection showed that early antibiotic therapy rapidly resolved hematologic and renal abnormalities (81). On the basis of available data, we suggest that in patients with Stx-*E. coli* gastrointestinal infection, antibiotics should be avoided unless in cases with sepsis.

A study with an Stx-binding agent, SYNSORB Pk, composed of particles of silicon linked to the globotriaosylceramide, given orally (82), failed to find any effect of SYNSORB over placebo. Most treatments, including plasma therapy, intravenous IgG, fibrinolytic agents, antiplatelet drugs, corticosteroids, and antioxidants (38), have been shown to be ineffective in controlled clinical trials in the acute phase of the disease (38). Careful BP control and renin-angiotensin system blockade may be particularly beneficial on the long term for patients who experience chronic renal disease after an episode of Stx-HUS. A recent study in 45 children who had renal sequelae of HUS and were followed for 9 to 11 yr documented that early restriction of proteins and use of angiotensin-converting enzyme inhibitors may have a beneficial effect on long-term renal outcome, as documented by a positive slope of 1/Cr values over time in treated patients (83). In another study, 8 to 15 yr of treatment with angiotensin-converting enzyme inhibitors after severe Stx-HUS normalized BP, reduced proteinuria, and improved GFR (84).

Finally, kidney transplant should be considered as an effective and safe treatment for children who progress to ESRD. Indeed, the outcome of renal transplantation is good in children with Stx-HUS: Recurrence rates range from 0 to 10% (85,86), and graft survival at 10 yr is even better than in control children who had other diseases and received a transplant (87).

Non–Stx-HUS

Epidemiology and Clinical Features

Non–Stx-HUS is less common than Stx-HUS and accounts for only 5 to 10% of all cases of the disease (1,88). It may manifest at all ages but is more frequent in adults. According to a recent U.S. study, the incidence of non–Stx-HUS in children is approximately one tenth that of Stx-HUS (10), corresponding to approximately 2 cases/yr per 1000,000 total population. At variance with Stx-HUS, there is no clear causative agent or seasonal pattern. The onset may be preceded by features of the nephrotic syndrome. A diarrhea prodrome is rarely observed (D^-HUS) (1,3,10,89). Non–Stx-HUS can occur sporadically or in families.

Sporadic Non–Stx-HUS. A wide variety of triggers for sporadic non–Stx-HUS have been identified, including various nonenteric infections, viruses, drugs, malignancies, transplantation, pregnancy, and other underlying medical conditions (scleroderma, antiphospholipid syndrome, lupus; Table 2). Infection caused by *Streptococcus pneumoniae* accounts for 40% of non–Stx-HUS and 4.7% of all causes of HUS in children in the United States (10). Neuroaminidase produced by *S. pneumoniae*, by removing sialic acids from the cell membranes, exposes Thomsen-Friedenreich antigen to preformed circulating IgM antibodies, which bind to this neoantigen on platelet and endothelial cells and cause platelet aggregation and endothelial damage (90,91). The clinical picture is usually severe, with respiratory distress, neurologic involvement, and coma and a mortality rate of 50% (91).

Categories of drugs that have been most frequently reported to induce non–Stx-HUS include anticancer molecules (mitomycin, cisplatin, bleomycin, and gemcitabine), immunotherapeutic (cyclosporine, tacrolimus, OKT3, IFN, and quinidine), and antiplatelet (ticlopidine and clopidogrel) agents (92). The risk for developing HUS after mitomycin is 2 to 10%. The onset is delayed, occurring almost 1 yr after starting treatment. The prognosis is poor, with up to 75% mortality at 4 mo (92).

Posttransplantation HUS is being reported with increasing frequency (1,93). It may ensue for the first time in patients who never experienced the disease (*de novo* posttransplantation HUS) or may affect patients whose primary cause of ESRD was HUS (recurrent posttransplantation HUS, discussed later in this review). *De novo* posttransplantation HUS might occur in patients who receive renal transplants and other organs, as a consequence of the use of calcineurin inhibitors or of humoral (C4b positive) rejection. It occurs in 5 to 15% of renal transplant patients who receive cyclosporine and in approximately 1% of those who are given tacrolimus (94).

Pregnancy-associated HUS may occasionally develop as a complication of preeclampsia. Some patients progress to a life-threatening variant of preeclampsia with severe thrombocytopenia, microangiopathic hemolytic anemia, renal failure, and liver involvement (HELLP syndrome). These forms are always an indication for prompt delivery that is usually followed by complete remission (95). Postpartum HUS manifests within 3 mo of delivery in most cases. The outcome is usually poor, with 50 to 60% mortality; residual renal dysfunction and hypertension are the rule in surviving patients (96). Of note, in approximately 50% of cases of sporadic non–Stx-HUS, no clear triggering conditions could be found (idiopathic HUS) (1).

Familial Non–Stx-HUS. Familial forms account for fewer than 3% of all cases of HUS. Both autosomal dominant and autosomal recessive forms of inheritance have been noted (97). In autosomal recessive HUS, the onset is usually early in childhood. The prognosis is poor, with a mortality rate of 60 to 70%. Recurrences are very frequent. Autosomal dominant HUS has an adult onset in most cases; the prognosis is poor, with a cumulative incidence of death or ESRD of 50 (98) to 90% (97).

Recent studies have documented that familial HUS may be

caused by genetic abnormalities of proteins involved in the regulation of the complement system. Similar genetic abnormalities have been found in sporadic non–Stx-HUS, mainly in idiopathic forms (99,100) but also in rare cases of pregnancy-associated (99) and postpartum HUS (three patients) (101,102), ticlopidine-induced HUS (one patient) (99), and postinfectious HUS (*Neisseria meningitidis*; one patient) (103).

Genetic Studies

Reduced serum levels of the third component (C3) of complement have been reported since 1974 in both familial and sporadic forms of non–Stx-HUS (98,104,105). Low C3 levels likely reflect C3 consumption in the microvasculature rather than defective synthesis, as documented by granular C3 deposits in glomeruli and arterioles of HUS patients (106,107) and by increased C3 breakdown products in sera. By contrast, levels of the fourth fraction of complement, C4, are usually normal (98). Persistent and remarkably depressed C3 levels found in patients with familial HUS, even in the unaffected relatives (98), suggested an inherited defect causing hyperactivation of the complement cascade.

The complement system consists of several plasma- and membrane-associated proteins that are organized in three activation pathways: The classical, the lectin, and the alternative pathway (108,109) (Figure 2). Upon activation by molecules on the surface of microorganisms, these pathways result in the formation of protease complexes, the C3 convertases, which cleave C3 generating C3b. The classic/lectin convertases are formed by C2 and C4 fragments, whereas the generation of the alternative pathway convertase requires the cleavage of C3 but not of C4. Thus, low C3 levels in patients with HUS in the presence of normal C4 indicate a selective activation of the alternative pathway (98).

Upon generation, C3b deposits on bacterial surfaces, which leads to opsonization for phagocytosis by PMN and macrophages. C3b also participates to the formation of the C5 convertases that cleaves C5 and initiates assembly of the membrane attack complex that causes cell lysis. The human complement system is highly regulated as to prevent nonspecific damage to host cells and limit deposition of C3b to the surface of pathogens. This fine regulation is based on a number of membrane-anchored (CR1, DAF, MCP, and CD59) and fluid-phase (factor H) regulators that protect host tissues. Foreign surfaces that either lack membrane-bound regulators or cannot bind soluble regulators are attacked by complement.

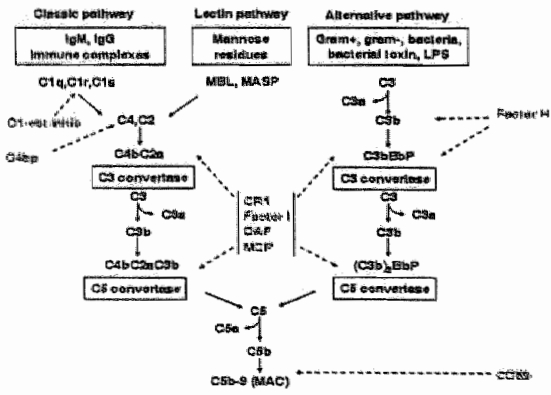

Figure 2. Activation pathways of the complement system and their regulators (in red).

In 1998, Warwicker *et al.* (110) studied three families with HUS and established linkage in the affected individuals to the regulator of complement activation gene cluster on human chromosome 1q32, which encodes for several complement regulatory proteins. The first examined candidate gene in this region was factor H (HF1), because an association between familial HUS and HF1 abnormalities had been reported previously (103,111,112). HF1 is a 150-kD multifunctional single-chain plasma glycoprotein that plays an important role in the regulation of the alternative pathway of complement (113). It serves as a co-factor for the C3b-cleaving enzyme factor I in the degradation of newly formed C3b molecules and controls decay, formation, and stability of the C3b convertase C3bBb. HF1 consists of 20 homologous units, named short consensus repeats (SCR). The complement regulatory domains that are needed to prevent fluid-phase alternative pathway amplification have been localized within the N-terminal SCR1–4 (114). The inactivation of surface-bound C3b is dependent on the binding of the C-terminal domain of HF1 to polyanionic molecules that increases HF1 affinity for C3b and exposes its complement regulatory N-terminal domain. The C-terminal domains contain two C3b binding sites, located in SCR12–14 and SCR19–20, and three polyanion-binding sites, located in SCR 7, SCR 13, and SCR19–20 (Figure 3) (115–117). However, the C3b and the polyanion-binding sites located in SCR19–20 are the only indispensable sites for HF1 to inactivate surface-bound C3b, because deletion of this portion of the molecule causes loss of HF1 capability to prevent complement activation on sheep erythrocytes (115,116). Human glomerular endothelial cells and kidney glomerular basement membrane are rich in polyanionic molecules, so HF1 deposited on their surface would provide an efficient shield against complement attack (Figure 4A) (117,118).

Since the first report by Warwicker, a number of studies have been performed by four independent groups, who altogether so far have identified up to 50 different HF1 mutations (Figure 3) in 80 patients who had familial (36 patients) and sporadic (44 patients) forms of non–Stx-HUS (99–102,114,119–121). In sporadic forms, the mutation was either inherited from a healthy parent or, more rarely—only four cases reported—ensued *de novo* in the proband (100,102). The mutation frequency is up to 40% in familial forms, whereas only 13 to 17% of sporadic forms had HF1 mutations (100,119). Alterations in other genes encoding for complement regulatory proteins could theoretically be involved in determining predisposition to sporadic non–Stx-HUS. Alternatively, these forms could be caused by an acquired autoimmune HF1 defect, similar to that observed in some patients with thrombotic thrombocytopenic purpura, in whom the acute episode is triggered by antibodies against the von

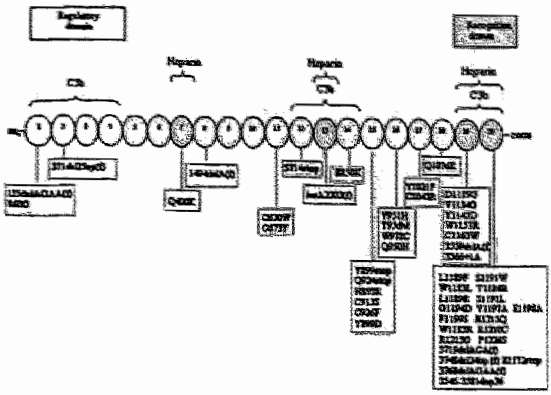

Figure 3. Factor H mutations associated with hemolytic uremic syndrome (HUS). The figure shows the structure of human factor H with the 20 short consensus repeats. The locations of the N-terminal regulatory domain responsible for co-factor activity and the binding sites for C3b and polyanions (heparin) are indicated. The majority of the mutations found in patients with HUS clusters in the C-terminus of factor H that is important for binding to polyanions and to surface-bound C3b and for the control of C3b deposition on cell membranes and extracellular matrix.

Willebrand factor cleaving metalloprotease ADAMTS-13 (122). This possibility is supported by a recently published paper (123) documenting the presence of anti-factor H antibodies in the plasma of three children with recurrent HUS.

The vast majority (48 of 50) of HF1 mutations in HUS patients are heterozygous and cause either single amino acid changes or premature translation interruption, mainly clustering in the C-terminus domains and are commonly associated with normal HF1 plasma levels. This is at variance with patients with type II membranoproliferative glomerulonephritis, who carry homozygous HF1 mutations that cause severely reduced HF1 levels (101). Expression and functional studies demonstrated that HF1 proteins that carry HUS-associated mutations have a severely reduced capability to interact with polyanions and with surface-bound C3b (117,118,124), which results in a lower density of mutant HF1 molecules bound to endothelial cells surface and a diminished complement regulatory activity on the cell membrane (117,118). In contrast, these mutants have a normal capacity to control activation of the complement in plasma, as indicated by data that they retain a normal co-factor activity in the proteolysis of fluid-phase C3b (124). The latter finding explains the case of patients who have HUS and HF1 mutations and normal serum complement levels (100,101). Sánchez-Corral *et al.* (125) proposed that HF1-related complement regulatory defects could be detected in patients' serum with an *ex vivo* hemolytic assay, in which serum from patients with HF1 mutations caused a more severe lysis of sheep erythrocytes than serum from patients without mutations. This, if confirmed, could represent a useful tool to select patients who have HUS and deserve studies of HF1 and other complement regulatory proteins.

Patients who carry HF1 mutations have a partial HF1 deficiency, as a result of one intact and one defective allele, which more likely predispose to rather than directly cause the disease. The observation that these patients occasionally have long remissions from HUS or do not present until late in life supports this hypothesis (125). In addition, conditions that trigger complement activation, either directly (bacterial and viral infections) or indirectly, by causing endothelial insult (drugs, systemic diseases, or pregnancy), precipitate the acute event in approximately 60% of patients with HF1 mutations (99,119). All of the above observations can be reconciled by reasoning that in these patients, the suboptimal HF1 activity is enough to protect the host from complement activation in physiologic conditions. However, upon exposure to an agent that activates complement, C3b is formed in higher-than-normal amounts, and its deposition on vascular endothelial cells cannot be fully prevented as a result of loss of polyanion binding capability of mutated HF1 (Figure 4B). This results in the formation of membrane attack complex and the recruitment of inflammatory cells, all events that cause damage and retraction of endothelial cells, adhesion and aggregation of platelets, increased local tissue factor with factor VII binding and activation, and the formation of thrombin and of fibrin polymers (Figure 4D). Such a scenario particularly applies to glomerular capillary bed, which is a fenestrated endothelium, and the exposed basement membrane supplies a surface that is rich in polyanions for HF1 binding, which could explain the renal localization of microvascular injury of HUS.

Two thirds of patients with non–Stx-HUS have no HF1 mutations, despite that up to 50% of them exhibit evidence of overactivity of the alternative pathway of complement (99). The possibility that uncommon polymorphic variants of HF1 gene may confer susceptibility to HUS in patients without HF1 mutations has been recently raised. Indeed, the T allele of the C-257T, the G allele of the A2089G, and the T allele of the G2881T polymorphisms were found to be more frequent in HUS patients without HF1 mutations than in healthy subjects (99), and analysis of the overall study population revealed that individuals who carry two or three of the above variants had a fourfold increased risk for developing HUS (99). The −257T, 2089G, and 2881T alleles might also have a role in determining the penetrance (which is approximately 50%) (99) of the disease in HF1 mutation carriers. In five of nine families, individuals who developed HUS had inherited an allele carrying the HF1 mutation from one parent together with an allele carrying at least one disease-associated HF1 polymorphism from the other parent. Instead, all of the healthy HF1 mutation carriers inherited only the mutation but no polymorphism (99).

Abnormalities in two additional genes encoding for complement modulatory proteins have also been involved recently in predisposition to non–Stx-HUS. Two reports from independent groups, published a few days apart, described mutations in MCP gene, encoding for membrane co-factor protein, a cell-bound complement regulator, in affected individuals of four families (127,128). MCP is a widely expressed transmembrane

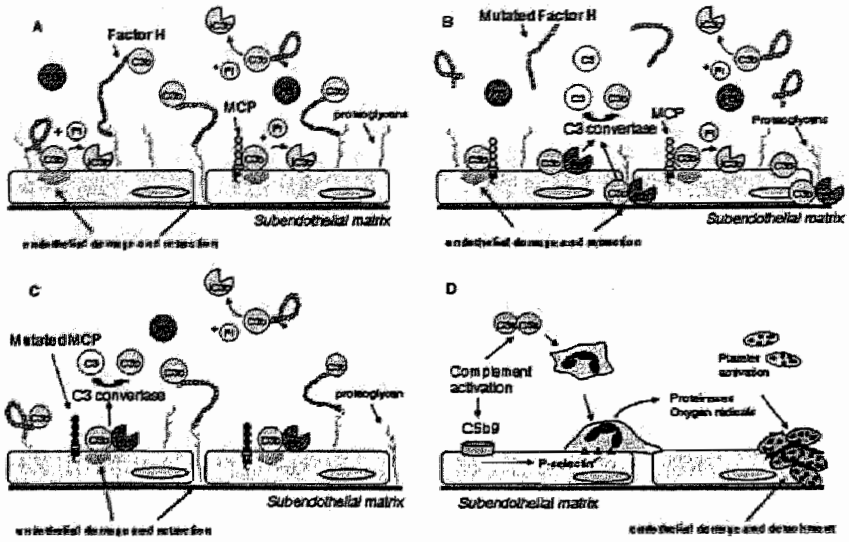

Figure 4. Proposed model for the pathologic consequences of factor H and monocyte chemoattractant protein (MCP) mutations. (A) After viral or bacterial infection or endothelial insult, complement is activated and C3b is formed. In the presence of normal factor H (HF1), C3b is rapidly inactivated to inactive C3b (iC3b). Factor H in the circulation binds fluid-phase C3b and favors its degradation by factor I (FI; co-factor activity, yellow domain). In addition, it binds (green domain) to polyanionic proteoglycans that are present on endothelial cell surface and in the subendothelial matrix, where, because of its high affinity for C3b, it entraps fluid-phase C3b, thus preventing its deposition on host surfaces and its binding with factor B (FB) to form the C3 convertase complex (C3bBb). The subendothelial matrix lacks endogenous complement regulators and is completely dependent on factor H to control complement activation. MCP also inactivates C3b deposited on endothelial cells by favoring its cleavage to iC3b by FI. (B) Proposed consequences of factor H mutations found in patients with HUS. Mutant factor H has a normal co-factor activity in fluid phase. However, the mutations affect the polyanion interaction site at the C-terminus of factor H so that it shows reduced bind to proteoglycans on endothelial cell surface and in subendothelial matrix. This results in more C3b reaching the endothelia cell surface so that MCP is not enough to control adequately complement activation on the cell membrane. In addition, C3b deposited on exposed extracellular matrix is not degraded and forms the C3 convertase of the alternative pathway of complement that further cleaves C3 to C3b. (C) MCP deficiency also predisposes to HUS. MCP mutations found in patients with HUS result in a reduced surface expression of the protein or in a reduced capability of MCP to bind C3b. In both cases, membrane-bound C3b is not efficiently inactivated, which leads to undesirable amplification of C3b formation and deposition on damaged endothelial cells through the formation of C3 convertase. (D) The sequence of events leading to microvascular thrombosis. The proteolysis of C3 and C5 by convertases causes the release of the chemotactic anaphylatoxins C3a and C5a that bind to receptors on inflammatory cells and attract them toward the endothelial layer. The deposition of C3b on endothelial cells is followed by the formation of the membrane attack complex (C5b9), which leads to cells' injury and detachment and to sublytic membrane perturbation, leading to endothelial activation and expression of adhesion molecules (*e.g.*, P-selectin). The latter favor leukocyte attachment and activation with the release of oxygen radicals and proteinases that further damage the endothelium. After endothelial damage, cell detachment ensues and exposes basement membranes. In these conditions, platelets from the microcirculation adhere and aggregate to the exposed matrix.

glycoprotein that serves as a co-factor for factor I to cleave C3b and C4b deposited on host cell surface (129–131). MCP has four extracellular complement control-protein modules (CCP) that are important for its inhibitory activity, followed by a serine-threonine-proline–rich domain, a transmembrane domain, and a cytoplasmic tail (132). Richards *et al.* (128) reported a heterozygous deletion of the D237/S238 amino acids in one family and a S206P substitution in two families. Evaluations of protein expression and function on PBMC showed that the mutants had a reduced C3b binding capability and a reduced ability to prevent complement activation. Another heterozygous mutation, causing two amino acid changes and a premature interruption of MCP protein in CCP4, was identified (127) in two siblings, which caused 50% reduction in MCP expression levels on PBMC of heterozygous individuals. Additional studies from our group on 112 patients with non–Stx-HUS have revealed five additional MCP mutations in familial (seven cases) and in sporadic (five cases) HUS with a mutation frequency of 11% (25% in familial and 6% in sporadic forms) (133).

MCP is highly expressed in the kidney and could be found on glomerular endothelial cells by immunohistochemical analysis (134–136). It likely exerts a main role in protecting glomerular endothelial cells against C3 activation as indicated by data that co-factor activity in the extracts of these cells was com-

pletely blocked by anti-MCP antibody (136). Factor H and MCP likely integrate each other in controlling complement activation on host cells. Polyanion-attached HF1 extends from the cell membrane by approximately 120 nm and could represent an outer barrier of cells against complement attack. However, because MCP is a small-sized membrane-integrated complement regulator that extends for approximately 20 nm, one can hypothesize that MCP protein is involved in the control of complement in the close vicinity of the cell membrane (117). As hypothesized for HF1, mutations in MCP likely predispose rather than directly cause HUS. Upon exposure to conditions that cause activation of the complement cascade, reduced levels or defective C3b binding capability and co-factor activity of mutated MCP on glomerular endothelial cells would result in an insufficient protection of these cells from complement activation (Figure 4C). That mutations either in factor H or in MCP result in complement activation and HUS indicates that these complement regulators do not have overlapping functions and that they both are necessary to control complement adequately.

Finally, three mutations in the gene encoding for factor I have been reported in three patients with sporadic non–Stx-HUS (137), which further support the concept that HUS is a disease of complement dysregulation. Other candidate genes are under investigation, including DAF, CR1, CD59, C3, and factor B.

Patients who present with non–Stx-HUS should be tested first for serum C3 concentrations; however, normal C3 levels do not necessarily exclude a complement dysfunction. More sensitive assays could be a higher-than-normal C3d/C3 ratio in plasma (unpublished data) or the presence of C3 deposits in renal biopsy (106,107). Measurement of HF1 in serum would be helpful to find out those few patients who carry HF1 mutations that cause reduced HF1 levels. Decreased CH50 values and factor B concentrations could be found in some but not all patients with HF1 or MCP mutations. A second step should be the search for mutations in candidate genes HF1 and MCP. Search for factor I mutations should be performed in patients with lower-than-normal factor I serum levels.

Which Treatment for Non–Stx-HUS?

Despite that non–Stx-HUS has a poor prognosis, after plasma manipulation was introduced, the mortality rate has dropped from 50 to 25% (138–140). However, debate still exists on whether plasma is or is not effective in the treatment of acute episodes (141–144). Published observations (139,145–147) and our own experience indicate that a consistent number of patients with non–Stx-HUS respond to plasma treatment. It has been proposed that plasma exchange might be relatively more effective than plasma infusion because it might remove potentially toxic substances from the patient's circulation. That this may not be the case is documented by data that in a patient with relapsing thrombotic microangiopathy (148), normalization of the platelet count was invariably obtained by plasma exchange or infusion, whereas plasma removal and substitution with albumin and saline never raised the platelet count. However, in situations such as renal insufficiency or heart failure, which limit the amount of plasma that can be provided with infusion alone, plasma exchange should be considered as first-choice therapy (1). Plasma treatment should be started within 24 h of presentation as delay in treatment initiation may increase treatment failure. Usually one plasma volume (40 ml/kg) is exchanged per session (1,149). Treatment can be intensified by increasing the volume of plasma replaced. The twice-daily exchanges of one plasma volume is probably the treatment of choice for refractory patients to minimize the recycling of infused plasma (1). As for plasma infusion, the recommended dose is 30 to 40 ml/kg on day 1, then 10 to 20 ml/kg per d. Daily plasma therapy should continue for a minimum of 2 d after complete remission is obtained (1,149).

Plasma infusion or exchange has been used in patients with HUS and HF1 mutations, with the rationale to provide the patients with normal HF1 to correct the genetic deficiency. Some patients did not respond at all and died or developed ESRD (107). Others remained chronically ill (121,150) or required infusion of plasma at weekly intervals to raise HF1 plasma levels enough to maintain remission (151). Stratton *et al.* (152) were able to induce sustained remission in a patient who had HF1 mutation and developed an acute episode of HUS and required hemodialysis. After 3 mo of weekly plasma exchange in conjunction with intravenous immunoglobulins, the patient regained renal function, dialysis was withdrawn, and plasma therapy was stopped. At 1 yr after stopping plasma therapy, the patient remained disease-free and dialysis independent. Plasma therapy is instead contraindicated in patients with HUS induced by *S. pneumoniae*, because adult plasma contains antibodies against the Thomsen-Friedenreich antigen, which may exacerbate the disease.

In those few patients with extensive microvascular thrombosis at renal biopsy, refractory hypertension, and signs of hypertensive encephalopathy, when conventional therapies including plasma manipulation are not enough to control the disease (*i.e.*, persistent severe thrombocytopenia and hemolytic anemia), bilateral nephrectomy has been performed with excellent follow-up in some patients (153). Other treatments, including antiplatelet agents, prostacyclin, heparin or fibrinolytic agents, steroids, and intravenous immunoglobulins, have been attempted, with no consistent benefit (1).

Patients who develop HUS upon challenge with cyclosporine or tacrolimus have to stop the medication. Sirolimus has been used as an alternative in occasional patients with encouraging results (154).

Of patients with non–Stx-HUS, 50% (in sporadic forms) to 60% (in familial forms) progress to ESRD (1,99). Renal transplantation is not necessarily an option for non–Stx-HUS, at variance with Stx-HUS. Actually, approximately 50% of the patients who had a renal transplant had a recurrence of the disease in the grafted organ (86,155). Recurrences occur at a median time of 30 d after transplant (range, 0 d to 16 yr). There is no effective treatment of recurrences. Graft failure occurs in >90% of patients who experience recurrence, despite plasma infusion or plasma exchange, high-dose prednisone, and withdrawal of cyclosporine (1,86). Patients who lost the first kidney graft for recurrence should not receive another transplant. Live-related renal transplant should also be avoided in that it carries the additional risk to precipitate the disease onset in the healthy

donor relative as recently reported in two families (156). New knowledge from genetic studies will predict more accurately the risk for recurrence. In patients with HF1 mutations, the recurrence rate ranges from 30 to 100%, according to different surveys (99–101), and is significantly higher than in patients without HF1 mutations (99). In view of the fact that HF1 is a plasma protein mainly of liver origin, a kidney transplant does not correct the HF1 genetic defect (110,119).

Simultaneous kidney and liver transplant was performed in two young children with non–Stx-HUS and HF1 mutations, with the objective of correcting the genetic defect and preventing disease recurrences (157,158). However, for reasons that are currently under evaluation and that possibly involve an increased liver susceptibility to immune or ischemic injury related to uncontrolled complement activation, both cases that were treated with this procedure were complicated by premature irreversible liver failure. In the first published case (157), a humoral rejection of the liver graft manifested by the 26th day after transplantation; the patient had actually a high titer of antibodies to donor class I HLA. In a few days, the child developed hepatic encephalopathy and coma that recovered with a second, uneventful liver transplant (157). The second case was complicated by a fatal, primary nonfunction of the liver graft. Graft hypoperfusion, as a result of a sudden drop of arterial BP occurring soon after reperfusion, triggered severe ischemia/reperfusion damage and complement deposition in the liver, conceivably as the result of defective HF1 complement regulatory potential. Multiorgan failure was the final event resulting in the patient's death (158). Thus, despite its capacity of correcting the genetic defect, combined kidney and liver transplant for non–Stx-HUS associated with HF1 mutations should not be performed unless a patient is at imminent risk for life-threatening complications.

Kidney graft outcome is favorable in patients with MCP mutations as found in four patients who received a successful transplant and experienced no disease recurrence (128; unpublished data). In view of the fact MCP is a membrane-bound protein that is highly expressed in the kidney, a kidney graft would reasonably correct local MCP dysfunction. The graft, bearing wild-type MCP expressed on renal endothelial cell surface, should conceivably be protected from disease recurrence.

The Future

Research efforts are aimed at identifying more specific approaches that may interfere with the primary cause of microangiopathy in the different forms of HUS. In Stx-HUS, new agents that are targeted at preventing organ exposure to Stx are currently under evaluation (159). In mice, molecular decoys such as orally administered harmless recombinant bacteria that display an Stx receptor on the surface that in turn binds the toxin in the gut (160–162) have been used successfully. Another approach is to use Stx inhibitors, among them is STARFISH, an oligobivalent, water-soluble carbohydrate ligand that can simultaneously engage all five B subunits of the toxin, which might help to prevent toxin that already has entered the circulation from destroying kidney microvessels (163). Others have ameliorated disease in pigs by injection of toxin-neutralizing antibodies (164). Some investigators have focused on downstream events in the pathogenetic cascade. Thrombin blockade with lepirudin in a model of Stx-HUS in greyhound dogs had some beneficial effect on mortality (165), indicating that thrombin may be a critical factor in the pathogenesis of Stx-HUS. At present, prevention remains the main approach to decreasing the morbidity and mortality associated with Stx-*E. coli* infection. A multifaceted approach that includes novel ways of decreasing Stx-*E. coli* carrier rate in livestock and implementing a zero-tolerance policy for contaminated foods and beverages is required.

The discovery of mutations in three different complement regulatory genes provides enough evidence of the involvement of complement activation in the pathogenesis of non–Stx-HUS and indicates that complement inhibition could represent a therapeutic target in these patients. There are currently a number of companies with complement inhibitors in clinical or preclinical development (166). Pexelizumab and eculizumab, two humanized monoclonal antibodies directed against C5 that inhibit the activation of terminal complement components, have been developed recently. Administration of eculizumab to patients with paroxysmal nocturnal hemoglobinuria, a disease characterized by a genetic deficiency of surface proteins that protect hematopoietic cells against the attack by the complement system, reduced intravascular hemolysis, hemoglobinuria, and the need for transfusions (167). In a phase II clinical trial, administration of pexelizumab as adjunctive therapy in patients who had myocardial infarction and underwent primary percutaneous coronary intervention inhibited complement activation and significantly reduced mortality as compared with the placebo group, although the infarct size was not modified by the drug (168). Another complement-blocking approach under investigation in clinical studies is based on the use of soluble forms of the C3/C5 convertase inhibitor complement receptor 1 (CR1). Phase I and phase II clinical trials have shown that the soluble CR1 TP10, administered intravenously both before and during surgery, decreased complement activation and protected vascular function in infants who underwent cardiopulmonary bypass (169). In a randomized, multicenter, prospective study in 564 high-risk patients who underwent cardiac surgery on cardiopulmonary bypass, a bolus of TP10 given immediately before cardiopulmonary bypass significantly inhibited complement activity within 10 min, and this inhibition persisted for 3 d postoperatively (170).

It is hoped that the above complement inhibitors, once available to the market, will be useful in patients with non–Stx-HUS, to block complement-mediated kidney damage during the acute episode or to prevent recurrence after kidney transplantation. Complement inhibitors also theoretically could be of benefit to prevent complications, such primary liver nonfunction, in combined kidney and liver transplantation in patients with HF1 genetic defects.

For HUS associated with HF1 mutations, specific replacement therapies with recombinant HF1 could become a viable alternative to plasma treatment. Efforts are also ongoing to isolate plasma fractions enriched in HF1, which could allow

providing the patient with enough active molecules while minimizing the risk for allergy and fluid overload. It is hoped that advances in vector safety and transfection efficiency will soon render gene therapy a realistic option for these patients. Undergoing studies on other complement regulatory genes would help to clarify fully the molecular determinants underlying the pathogenesis of non–Stx-HUS and hopefully translate into an improvement in the management and therapy.

Acknowledgments

This work was partially supported by grants from Comitato 30 ore per la vita, from Telethon (grants GPP02161 and GPP02162), and from the Foundation for Children with Atypical HUS along with The Nando Peretti Foundation.

References

1. Ruggenenti P, Noris M, Remuzzi G: Thrombotic microangiopathy, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura. *Kidney Int* 60: 831–846, 2001
2. Gianviti A, Rosmini F, Caprioli A, Corona R, Matteucci MC, Principato F, Luzzi I, Rizzoni G: Haemolytic-uraemic syndrome in childhood: Surveillance and case-control studies in Italy. Italian HUS Study Group. *Pediatr Nephrol* 8: 705–709, 1994
3. Kaplan BS, Meyers KE, Schulman SL: The pathogenesis and treatment of hemolytic uremic syndrome. *J Am Soc Nephrol* 9: 1126–1133, 1998
4. Tonshoff B, Sammet A, Sanden I, Mehls O, Waldherr R, Scharer K: Outcome and prognostic determinants in the hemolytic uremic syndrome of children. *Nephron* 68: 63–70, 1994
5. Banatvala N, Griffin PM, Greene KD, Barrett TJ, Bibb WF, Green JH, Wells JG: The United States National Prospective Hemolytic Uremic Syndrome Study: Microbiologic, serologic, clinical, and epidemiologic findings. *J Infect Dis* 183: 1063–1070, 2001
6. Milford D: The hemolytic uremic syndromes in the United Kingdom. In: *Hemolytic Uremic Syndrome and Thrombotic Thrombocytopenic Purpura*, edited by Kaplan BS, Trompeter RS, Moake JL, New York, Marcel Dekker, 1992, pp 39–59
7. Van Dyck M, Proesmans W, Depraetere M: Hemolytic uremic syndrome in childhood: Renal function ten years later. *Clin Nephrol* 29: 109–112, 1988
8. Schieppati A, Ruggenenti P, Cornejo RP, Ferrario F, Gregorini G, Zucchelli P, Rossi E, Remuzzi G: Renal function at hospital admission as a prognostic factor in adult hemolytic uremic syndrome. The Italian Registry of Haemolytic Uremic Syndrome. *J Am Soc Nephrol* 2: 1640–1644, 1992
9. Taylor CM, Chua C, Howie AJ, Risdon RA: Clinico-pathological findings in diarrhoea-negative haemolytic uraemic syndrome. *Pediatr Nephrol* 19: 419–425, 2004
10. Constantinescu AR, Bitzan M, Weiss LS, Christen E, Kaplan BS, Cnaan A, Trachtman H: Non-enteropathic hemolytic uremic syndrome: Causes and short-term course. *Am J Kidney Dis* 43: 976–982, 2004
11. Taylor CM, Howie AJ, Williams JM: No common final pathogenetic pathway in haemolytic uraemic syndromes. *Nephrol Dial Transplant* 14: 1100–1102, 1999
12. Remuzzi G, Ruggenenti P: Thrombotic microangiopathies. In: *Renal Pathology*, 2nd Ed., edited by Tisher C, Brenner B, Philadelphia, J.B. Lippincott, 1994, pp 1154–1184
13. Thorpe CM: Shiga toxin-producing *Escherichia coli* infection. *Clin Infect Dis* 38: 1298–1303, 2004
14. Karmali MA, Steele BT, Petric M, Lim C: Sporadic cases of haemolytic-uraemic syndrome associated with faecal cytotoxin and cytotoxin-producing *Escherichia coli* in stools. *Lancet* 1: 619–620, 1983
15. Riley LW, Remis RS, Helgerson SD, McGee HB, Wells JG, Davis BR, Hebert RJ, Olcott ES, Johnson LM, Hargrett NT, Blake PA, Cohen ML: Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *N Engl J Med* 308: 681–685, 1983
16. Brooks JT, Bergmire-Sweat D, Kennedy M, Hendricks K, Garcia M, Marengo L, Wells J, Ying M, Bibb W, Griffin PM, Hoekstra RM, Friedman CR: Outbreak of Shiga toxin-producing *Escherichia coli* O111:H8 infections among attendees of a high school cheerleading camp. *Clin Infect Dis* 38: 190–198, 2004
17. Varma JK, Greene KD, Reller ME, DeLong SM, Trottier J, Nowicki SF, DiOrio M, Koch EM, Bannerman TL, York ST, Lambert-Fair MA, Wells JG, Mead PS: An outbreak of *Escherichia coli* O157 infection following exposure to a contaminated building. *JAMA* 290: 2709–2712, 2003
18. Caprioli A, Luzzi I, Rosmini F, Pasquini P, Cirrincione R, Gianviti A, Matteucci MC, Rizzoni G: Hemolytic-uremic syndrome and Vero cytotoxin-producing *Escherichia coli* infection in Italy. The HUS Italian Study Group. *J Infect Dis* 166: 154–158, 1992
19. Ludwig K, Bitzan M, Zimmermann S, Kloth M, Ruder H, Muller-Wiefel DE: Immune response to non-O157 Vero toxin-producing *Escherichia coli* in patients with hemolytic uremic syndrome. *J Infect Dis* 174: 1028–1039, 1996
20. Farmer JJ 3rd, Davis BR: H7 antiserum-sorbitol fermentation medium: A single tube screening medium for detecting *Escherichia coli* O157:H7 associated with hemorrhagic colitis. *J Clin Microbiol* 22: 620–625, 1985
21. McCarthy TA, Barrett NL, Hadler JL, Salsbury B, Howard RT, Dingman DW, Brinkman CD, Bibb WF, Cartter ML: Hemolytic-uremic syndrome and *Escherichia coli* O121 at a lake in Connecticut, 1999. *Pediatrics* 108: E59, 2001
22. Sonntag AK, Prager R, Bielaszewska M, Zhang W, Fruth A, Tschape H, Karch H: Phenotypic and genotypic analyses of enterohemorrhagic *Escherichia coli* O145 strains from patients in Germany. *J Clin Microbiol* 42: 954–962, 2004
23. Lopez EL, Diaz M, Grinstein S, Devoto S, Mendilaharzu F, Murray BE, Ashkenazi S, Rubeglio E, Woloj M, Vasquez M, *et al.*: Hemolytic uremic syndrome and diarrhea in Argentine children: The role of Shiga-like toxins. *J Infect Dis* 160: 469–475, 1989
24. Srivastava RN, Moudgil A, Bagga A, Vasudev AS: Hemolytic uremic syndrome in children in northern India. *Pediatr Nephrol* 5: 284–288, 1991
25. Guerin PJ, Brasher C, Baron E, Mic D, Grimont F, Ryan M, Aavitsland P, Legros D: *Shigella dysenteriae* serotype 1 in west Africa: Intervention strategy for an outbreak in Sierra Leone. *Lancet* 362: 705–706, 2003
26. Houdouin V, Doit C, Mariani P, Brahimi N, Loirat C, Bourrillon A, Bingen E: A pediatric cluster of *Shigella dysenteriae* serotype 1 diarrhea with hemolytic uremic syndrome in 2 families from France. *Clin Infect Dis* 38: e96–99, 2004

27. Mead PS, Griffin PM: *Escherichia coli* O157:H7. *Lancet* 352: 1207–1212, 1998
28. Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, Griffin PM, Tauxe RV: Food-related illness and death in the United States. *Emerg Infect Dis* 5: 607–625, 1999
29. Cleary TG: Cytotoxin-producing *Escherichia coli* and the hemolytic uremic syndrome. *Pediatr Clin North Am* 35: 485–501, 1988
30. Lopez EL, Prado-Jimenez V, O'Ryan-Gallardo M, Contrini MM: Shigella and Shiga toxin-producing *Escherichia coli* causing bloody diarrhea in Latin America. *Infect Dis Clin North Am* 14: 41–65, viii, 2000
31. Meichtri L, Miliwebsky E, Gioffre A, Chinen I, Baschkier A, Chillemi G, Guth BE, Masana MO, Cataldi A, Rodriguez HR, Rivas M: Shiga toxin-producing *Escherichia coli* in healthy young beef steers from Argentina: Prevalence and virulence properties. *Int J Food Microbiol* 96: 189–198, 2004
32. Griffin PM, Tauxe RV: The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. *Epidemiol Rev* 13: 60–98, 1991
33. Locking ME, O'Brien SJ, Reilly WJ, Wright EM, Campbell DM, Coia JE, Browning LM, Ramsay CN: Risk factors for sporadic cases of *Escherichia coli* O157 infection: The importance of contact with animal excreta. *Epidemiol Infect* 127: 215–220, 2001
34. Mead PS, Finelli L, Lambert-Fair MA, Champ D, Townes J, Hutwagner L, Barrett T, Spitalny K, Mintz E: Risk factors for sporadic infection with *Escherichia coli* O157:H7. *Arch Intern Med* 157: 204–208, 1997
35. Cody SH, Glynn MK, Farrar JA, Cairns KL, Griffin PM, Kobayashi J, Fyfe M, Hoffman R, King AS, Lewis JH, Swaminathan B, Bryant RG, Vugia DJ: An outbreak of *Escherichia coli* O157:H7 infection from unpasteurized commercial apple juice. *Ann Intern Med* 130: 202–209, 1999
36. Chandler WL, Jelacic S, Boster DR, Ciol MA, Williams GD, Watkins SL, Igarashi T, Tarr PI: Prothrombotic coagulation abnormalities preceding the hemolytic-uremic syndrome. *N Engl J Med* 346: 23–32, 2002
37. Beatty ME, Griffin PM, Tulu AN, Olsen SJ: Culturing practices and antibiotic use in children with diarrhea. *Pediatrics* 113: 628–629, 2004
38. Garg AX, Suri RS, Barrowman N, Rehman F, Matsell D, Rosas-Arellano MP, Salvadori M, Haynes RB, Clark WF: Long-term renal prognosis of diarrhea-associated hemolytic uremic syndrome: A systematic review, meta-analysis, and meta-regression. *JAMA* 290: 1360–1370, 2003
39. Date A, Raghupathy P, Jadhav M, Pereira SM, Shastry JC: Outcome of the haemolytic-uraemic syndrome complicating bacillary dysentery. *Ann Trop Paediatr* 2: 1–6, 1982
40. Adam A: *Jahrb F Kinderh* 116, 1927
41. Giles G, Sangster G, Smith J: Epidemic gastroenteritis of infants in Aberdeen in 1947. *Arch Intern Med* 24: 45–51, 1949
42. Belnap W, O'Donnell J: Epidemic gastroenteritis due to *Escherichia coli* O-111. *J Pediatr* 47: 178–183, 1955
43. Konowalchuk J, Speirs JI, Stavric S: Vero response to a cytotoxin of *Escherichia coli*. *Infect Immun* 18: 775–779, 1977
44. Jackson MP, Newland JW, Holmes RK, O'Brien AD: Nucleotide sequence analysis of the structural genes for Shiga-like toxin I encoded by bacteriophage 933J from *Escherichia coli*. *Microb Pathog* 2: 147–153, 1987
45. Tesh VL, Burris JA, Owens JW, Gordon VM, Wadolkowski EA, O'Brien AD, Samuel JE: Comparison of the relative toxicities of Shiga-like toxins type I and type II for mice. *Infect Immun* 61: 3392–3402, 1993
46. Fraser ME, Fujinaga M, Cherney MM, Melton-Celsa AR, Twiddy EM, O'Brien AD, James MN: Structure of Shiga toxin type 2 (Stx2) from *Escherichia coli* O157:H7. *J Biol Chem* 279: 27511–27517, 2004
47. Scotland SM, Willshaw GA, Smith HR, Rowe B: Properties of strains of *Escherichia coli* belonging to serogroup O157 with special reference to production of Vero cytotoxins VT1 and VT2. *Epidemiol Infect* 99: 613–624, 1987
48. Ostroff SM, Kobayashi JM, Lewis JH: Infections with *Escherichia coli* O157:H7 in Washington State. The first year of statewide disease surveillance. *JAMA* 262: 355–359, 1989
49. Cimolai N, Carter JE, Morrison BJ, Anderson JD: Risk factors for the progression of *Escherichia coli* O157:H7 enteritis to hemolytic-uremic syndrome. *J Pediatr* 116: 589–592, 1990
50. Jenkins C, Willshaw GA, Evans J, Cheasty T, Chart H, Shaw DJ, Dougan G, Frankel G, Smith HR: Subtyping of virulence genes in verocytotoxin-producing *Escherichia coli* (VTEC) other than serogroup O157 associated with disease in the United Kingdom. *J Med Microbiol* 52: 941–947, 2003
51. Siegler RL, Obrig TG, Pysher TJ, Tesh VL, Denkers ND, Taylor FB: Response to Shiga toxin 1 and 2 in a baboon model of hemolytic uremic syndrome. *Pediatr Nephrol* 18: 92–96, 2003
52. Fraser ME, Chernaia MM, Kozlov YV, James MN: Crystal structure of the holotoxin from *Shigella dysenteriae* at 2.5: A resolution. *Nat Struct Biol* 1: 59–64, 1994
53. Paton AW, Srimanote P, Talbot UM, Wang H, Paton JC: A new family of potent AB5 cytotoxins produced by Shiga toxigenic *Escherichia coli*. *J Exp Med* 200: 35–46, 2004
54. Donnenberg MS, Tacket CO, James SP, Losonsky G, Nataro JP, Wasserman SS, Kaper JB, Levine MM: Role of the eaeA gene in experimental enteropathogenic *Escherichia coli* infection. *J Clin Invest* 92: 1412–1417, 1993
55. Acheson DW, Moore R, De Breucker S, Lincicome L, Jacewicz M, Skutelsky E, Keusch GT: Translocation of Shiga toxin across polarized intestinal cells in tissue culture. *Infect Immun* 64: 3294–3300, 1996
56. Hurley BP, Thorpe CM, Acheson DW: Shiga toxin translocation across intestinal epithelial cells is enhanced by neutrophil transmigration. *Infect Immun* 69: 6148–6155, 2001
57. Bitzan M, Richardson S, Huang C, Boyd B, Petric M, Karmali MA: Evidence that verotoxins (Shiga-like toxins) from *Escherichia coli* bind to P blood group antigens of human erythrocytes in vitro. *Infect Immun* 62: 3337–3347, 1994
58. Cooling LL, Walker KE, Gille T, Koerner TA: Shiga toxin binds human platelets via globotriaosylceramide (Pk antigen) and a novel platelet glycosphingolipid. *Infect Immun* 66: 4355–4366, 1998
59. van Setten PA, Monnens LA, Verstraten RG, van den Heuvel LP, van Hinsbergh VW: Effects of verocytotoxin-1 on nonadherent human monocytes: Binding characteristics, protein synthesis, and induction of cytokine release. *Blood* 88: 174–183, 1996
60. te Loo DM, Monnens LA, van Der Velden TJ, Vermeer MA, Preyers F, Demacker PN, van Den Heuvel LP, van Hinsbergh VW: Binding and transfer of verocytotoxin by polymorphonuclear leukocytes in hemolytic uremic syndrome. *Blood* 95: 3396–3402, 2000

61. te Loo DM, van Hinsbergh VW, van den Heuvel LP, Monnens LA: Detection of verocytotoxin bound to circulating polymorphonuclear leukocytes of patients with hemolytic uremic syndrome. *J Am Soc Nephrol* 12: 800–806, 2001
62. Nakajima H, Kiyokawa N, Katagiri YU, Taguchi T, Suzuki T, Sekino T, Mimori K, Ebata T, Saito M, Nakao H, Takeda T, Fujimoto J: Kinetic analysis of binding between Shiga toxin and receptor glycolipid Gb3Cer by surface plasmon resonance. *J Biol Chem* 276: 42915–42922, 2001
63. Louise CB, Obrig TG: Specific interaction of *Escherichia coli* O157:H7-derived Shiga-like toxin II with human renal endothelial cells. *J Infect Dis* 172: 1397–1401, 1995
64. Ohmi K, Kiyokawa N, Takeda T, Fujimoto J: Human microvascular endothelial cells are strongly sensitive to Shiga toxins. *Biochem Biophys Res Commun* 251: 137–141, 1998
65. Obrig TG, Louise CB, Lingwood CA, Boyd B, Barley-Maloney L, Daniel TO: Endothelial heterogeneity in Shiga toxin receptors and responses. *J Biol Chem* 268: 15484–15488, 1993
66. van Setten PA, van Hinsbergh VW, van der Velden TJ, van de Kar NC, Vermeer M, Mahan JD, Assmann KJ, van den Heuvel LP, Monnens LA: Effects of TNF alpha on verocytotoxin cytotoxicity in purified human glomerular microvascular endothelial cells. *Kidney Int* 51: 1245–1256, 1997
67. Obrig TG, Moran TP, Brown JE: The mode of action of Shiga toxin on peptide elongation of eukaryotic protein synthesis. *Biochem J* 244: 287–294, 1987
68. Pijpers AH, van Setten PA, van den Heuvel LP, Assmann KJ, Dijkman HB, Pennings AH, Monnens LA, van Hinsbergh VW: Verocytotoxin-induced apoptosis of human microvascular endothelial cells. *J Am Soc Nephrol* 12: 767–778, 2001
69. Brigotti M, Alfieri R, Sestili P, Bonelli M, Petronini PG, Guidarelli A, Barbieri L, Stirpe F, Sperti S: Damage to nuclear DNA induced by Shiga toxin 1 and ricin in human endothelial cells. *FASEB J* 16: 365–372, 2002
70. Erwert RD, Eiting KT, Tupper JC, Winn RK, Harlan JM, Bannerman DD: Shiga toxin induces decreased expression of the anti-apoptotic protein Mcl-1 concomitant with the onset of endothelial apoptosis. *Microb Pathog* 35: 87–93, 2003
71. Zoja C, Angioletti S, Donadelli R, Zanchi C, Tomasoni S, Binda E, Imberti B, te Loo M, Monnens L, Remuzzi G, Morigi M: Shiga toxin-2 triggers endothelial leukocyte adhesion and transmigration via NF-κB dependent up-regulation of IL-8 and MCP-1. *Kidney Int* 62: 846–856, 2002
72. Matussek A, Lauber J, Bergau A, Hansen W, Rohde M, Dittmar KE, Gunzer M, Mengel M, Gatzlaff P, Hartmann M, Buer J, Gunzer F: Molecular and functional analysis of Shiga toxin-induced response patterns in human vascular endothelial cells. *Blood* 102: 1323–1332, 2003
73. Morigi M, Micheletti G, Figliuzzi M, Imberti B, Karmali MA, Remuzzi A, Remuzzi G, Zoja C: Verotoxin-1 promotes leukocyte adhesion to cultured endothelial cells under physiologic flow conditions. *Blood* 86: 4553–4558, 1995
74. Morigi M, Galbusera M, Binda E, Imberti B, Gastoldi S, Remuzzi A, Zoja C, Remuzzi G: Verotoxin-1-induced up-regulation of adhesive molecules renders microvascular endothelial cells thrombogenic at high shear stress. *Blood* 98: 1828–1835, 2001
75. Ruggeri Z: Endothelial cells: They only look all alike. *Blood* 98: 1644, 2001
76. Bergstein JM, Riley M, Bang NU: Role of plasminogen-activator inhibitor type 1 in the pathogenesis and outcome of the hemolytic uremic syndrome. *N Engl J Med* 327: 755–759, 1992
77. Wong CS, Jelacic S, Habeeb RL, Watkins SL, Tarr PI: The risk of the hemolytic-uremic syndrome after antibiotic treatment of *Escherichia coli* O157:H7 infections. *N Engl J Med* 342: 1930–1936, 2000
78. Safdar N, Said A, Gangnon RE, Maki DG: Risk of hemolytic uremic syndrome after antibiotic treatment of *Escherichia coli* O157:H7 enteritis: A meta-analysis. *JAMA* 288: 996–1001, 2002
79. Bhimma R, Rollins NC, Coovadia HM, Adhikari M: Post-dysenteric hemolytic uremic syndrome in children during an epidemic of Shigella dysentery in Kwazulu/Natal. *Pediatr Nephrol* 11: 560–564, 1997
80. Oneko M, Nyathi MN, Doehring E: Post-dysenteric hemolytic uremic syndrome in Bulawayo, Zimbabwe. *Pediatr Nephrol* 16: 1142–1145, 2001
81. Chiurchiu C, Firrincieli A, Santostefano M, Fusaroli M, Remuzzi G, Ruggenenti P: Adult nondiarrhea hemolytic uremic syndrome associated with Shiga toxin *Escherichia coli* O157:H7 bacteremia and urinary tract infection. *Am J Kidney Dis* 41: E4, 2003
82. Trachtman H, Cnaan A, Christen E, Gibbs K, Zhao S, Acheson DW, Weiss R, Kaskel FJ, Spitzer A, Hirschman GH: Effect of an oral Shiga toxin-binding agent on diarrhea-associated hemolytic uremic syndrome in children: A randomized controlled trial. *JAMA* 290: 1337–1344, 2003
83. Caletti MG, Lejarraga H, Kelmansky D, Missoni M: Two different therapeutic regimes in patients with sequelae of hemolytic-uremic syndrome. *Pediatr Nephrol* 19: 1148–1152, 2004
84. Van Dyck M, Proesmans W: Renoprotection by ACE inhibitors after severe hemolytic uremic syndrome. *Pediatr Nephrol* 19: 688–690, 2004
85. Loirat C, Niaudet P: The risk of recurrence of hemolytic uremic syndrome after renal transplantation in children. *Pediatr Nephrol* 18: 1095–1101, 2003
86. Artz MA, Steenbergen EJ, Hoitsma AJ, Monnens LA, Wetzels JF: Renal transplantation in patients with hemolytic uremic syndrome: High rate of recurrence and increased incidence of acute rejections. *Transplantation* 76: 821–826, 2003
87. Ferraris JR, Ramirez JA, Ruiz S, Caletti MG, Vallejo G, Piantanida JJ, Araujo JL, Sojo ET: Shiga toxin-associated hemolytic uremic syndrome: Absence of recurrence after renal transplantation. *Pediatr Nephrol* 17: 809–814, 2002
88. Hall SM, Glickman M: The British Paediatric Surveillance Unit. *Arch Intern Med* 63: 344–346, 1988
89. Renaud C, Niaudet P, Gagnadoux MF, Broyer M, Habib R: Haemolytic uraemic syndrome: Prognostic factors in children over 3 years of age. *Pediatr Nephrol* 9: 24–29, 1995
90. Erickson LC, Smith WS, Biswas AK, Camarca MA, Waecker NJ Jr: *Streptococcus pneumoniae*-induced hemolytic uremic syndrome: A case for early diagnosis. *Pediatr Nephrol* 8: 211–213, 1994
91. Novak RW, Martin CR, Orsini EN: Hemolytic-uremic syndrome and T-cryptantigen exposure by neuraminidase-producing pneumococci: An emerging problem? *Pediatr Pathol* 1: 409–413, 1983
92. Dlott JS, Danielson CF, Blue-Hnidy DE, McCarthy LJ:

Drug-induced thrombotic thrombocytopenic purpura/hemolytic uremic syndrome: A concise review. *Ther Apher Dial* 8: 102–111, 2004
93. Reynolds JC, Agodoa LY, Yuan CM, Abbott KC: Thrombotic microangiopathy after renal transplantation in the United States. *Am J Kidney Dis* 42: 1058–1068, 2003
94. Ruggenenti P: Post-transplant hemolytic-uremic syndrome. *Kidney Int* 62: 1093–1104, 2002
95. Remuzzi G, Ruggenenti P: The hemolytic uremic syndrome. *Kidney Int* 48: 2–19, 1995
96. George JN: The association of pregnancy with thrombotic thrombocytopenic purpura-hemolytic uremic syndrome. *Curr Opin Hematol* 10: 339–344, 2003
97. Berns JS, Kaplan BS, Mackow RC, Hefter LG: Inherited hemolytic uremic syndrome in adults. *Am J Kidney Dis* 19: 331–334, 1992
98. Noris M, Ruggenenti P, Perna A, Orisio S, Caprioli J, Skerka C, Vasile B, Zipfel PF, Remuzzi G: Hypocomplementemia discloses genetic predisposition to hemolytic uremic syndrome and thrombotic thrombocytopenic purpura: Role of factor H abnormalities. Italian Registry of Familial and Recurrent Hemolytic Uremic Syndrome/Thrombotic Thrombocytopenic Purpura. *J Am Soc Nephrol* 10: 281–293, 1999
99. Caprioli J, Castelletti F, Bucchioni S, Bettinaglio P, Bresin E, Pianetti G, Gamba S, Brioschi S, Daina E, Remuzzi G, Noris M: Complement factor H mutations and gene polymorphisms in haemolytic uraemic syndrome: The C-257T, the A2089G and the G2881T polymorphisms are strongly associated with the disease. *Hum Mol Genet* 12: 3385–3395, 2003
100. Neumann HP, Salzmann M, Bohnert-Iwan B, Mannuelian T, Skerka C, Lenk D, Bender BU, Cybulla M, Riegler P, Konigsrainer A, Neyer U, Bock A, Widmer U, Male DA, Franke G, Zipfel PF: Haemolytic uraemic syndrome and mutations of the factor H gene: A registry-based study of German speaking countries. *J Med Genet* 40: 676–681, 2003
101. Dragon-Durey MA, Fremeaux-Bacchi V, Loirat C, Blouin J, Niaudet P, Deschenes G, Coppo P, Herman Fridman W, Weiss L: Heterozygous and homozygous factor h deficiencies associated with hemolytic uremic syndrome or membranoproliferative glomerulonephritis: Report and genetic analysis of 16 cases. *J Am Soc Nephrol* 15: 787–795, 2004
102. Perez-Caballero D, Gonzalez-Rubio C, Gallardo ME, Vera M, Lopez-Trascasa M, Rodriguez de Cordoba S, Sanchez-Corral P: Clustering of missense mutations in the C-terminal region of factor H in atypical hemolytic uremic syndrome. *Am J Hum Genet* 68: 478–484, 2001
103. Rougier N, Kazatchkine MD, Rougier JP, Fremeaux-Bacchi V, Blouin J, Deschenes G, Soto B, Baudouin V, Pautard B, Proesmans W, Weiss E, Weiss L: Human complement factor H deficiency associated with hemolytic uremic syndrome. *J Am Soc Nephrol* 9: 2318–2326, 1998
104. Stuhlinger W, Kourilsky O, Kanfer A, Sraer JD: Haemolytic-uraemic syndrome: Evidence for intravascular C3 activation [Letter]. *Lancet* 2: 788–789, 1974
105. Carreras L, Romero R, Requesens C, Oliver AJ, Carrera M, Clavo M, Alsina J: Familial hypocomplementemic hemolytic uremic syndrome with HLA-A3,B7 haplotype. *JAMA* 245: 602–604, 1981
106. Hammar SP, Bloomer HA, McCloskey D: Adult hemolytic uremic syndrome with renal arteriolar deposition of IgM and C3. *Am J Clin Pathol* 70: 434–439, 1978
107. Landau D, Shalev H, Levy-Finer G, Polonsky A, Segev Y, Katchko L: Familial hemolytic uremic syndrome associated with complement factor H deficiency. *J Pediatr* 138: 412–417, 2001
108. Walport MJ: Complement. Second of two parts. *N Engl J Med* 344: 1140–1144, 2001
109. Walport MJ: Complement. First of two parts. *N Engl J Med* 344: 1058–1066, 2001
110. Warwicker P, Goodship TH, Donne RL, Pirson Y, Nicholls A, Ward RM, Turnpenny P, Goodship JA: Genetic studies into inherited and sporadic hemolytic uremic syndrome. *Kidney Int* 53: 836–844, 1998
111. Pichette V, Querin S, Schurch W, Brun G, Lehner-Netsch G, Delage JM: Familial hemolytic-uremic syndrome and homozygous factor H deficiency. *Am J Kidney Dis* 24: 936–941, 1994
112. Thompson RA, Winterborn MH: Hypocomplementaemia due to a genetic deficiency of beta 1H globulin. *Clin Exp Immunol* 46: 110–119, 1981
113. Zipfel PF, Skerka C: Complement factor H and related proteins: An expanding family of complement-regulatory proteins? *Immunol Today* 15: 121–126, 1994
114. Rodriguez de Cordoba S, Esparza-Gordillo J, Goicoechea de Jorge E, Lopez-Trascasa M, Sanchez-Corral P: The human complement factor H: Functional roles, genetic variations and disease associations. *Mol Immunol* 41: 355–367, 2004
115. Pangburn MK: Cutting edge: Localization of the host recognition functions of complement factor H at the carboxyl-terminal: Implications for hemolytic uremic syndrome. *J Immunol* 169: 4702–4706, 2002
116. Hellwage J, Jokiranta TS, Friese MA, Wolk TU, Kampen E, Zipfel PF, Meri S: Complement C3b/C3d and cell surface polyanions are recognized by overlapping binding sites on the most carboxyl-terminal domain of complement factor H. *J Immunol* 169: 6935–6944, 2002
117. Jozsi M, Manuelian T, Heinen S, Oppermann M, Zipfel PF: Attachment of the soluble complement regulator factor H to cell and tissue surfaces: Relevance for pathology. *Histol Histopathol* 19: 251–258, 2004
118. Manuelian T, Hellwage J, Meri S, Caprioli J, Noris M, Heinen S, Jozsi M, Neumann HP, Remuzzi G, Zipfel PF: Mutations in factor H reduce binding affinity to C3b and heparin and surface attachment to endothelial cells in hemolytic uremic syndrome. *J Clin Invest* 111: 1181–1190, 2003
119. Caprioli J, Bettinaglio P, Zipfel PF, Amadei B, Daina E, Gamba S, Skerka C, Marziliano N, Remuzzi G, Noris M: The molecular basis of familial hemolytic uremic syndrome: Mutation analysis of factor H gene reveals a hot spot in short consensus repeat 20. *J Am Soc Nephrol* 12: 297–307, 2001
120. Buddles MR, Donne RL, Richards A, Goodship J, Goodship TH: Complement factor H gene mutation associated with autosomal recessive atypical hemolytic uremic syndrome. *Am J Hum Genet* 66: 1721–1722, 2000
121. Filler G, Radhakrishnan S, Strain L, Hill A, Knoll G, Goodship TH: Challenges in the management of infantile factor H associated hemolytic uremic syndrome. *Pediatr Nephrol* 19: 908–911, 2004

122. Tsai HM, Lian EC: Antibodies to von Willebrand factor-cleaving protease in acute thrombotic thrombocytopenic purpura. *N Engl J Med* 339: 1585–1594, 1998
123. Brioschi S, Porrati F, Bresin E, Todeschini M, Bucchioni S, Pianetti G, Castelletti F, Caprioli J, Remuzzi G, Noris M: Mutations in membrane cofactor protein in atypical hemolytic uremic syndrome [Abstract]. Presented at the 7th Congress of the Italian Society of Human Genomics, October 13–15, 2004
124. Sanchez-Corral P, Perez-Caballero D, Huarte O, Simckes AM, Goicoechea E, Lopez-Trascasa M, de Cordoba SR: Structural and functional characterization of factor H mutations associated with atypical hemolytic uremic syndrome. *Am J Hum Genet* 71: 1285–1295, 2002
125. Sanchez-Corral P, Gonzalez-Rubio C, Rodriguez de Cordoba S, Lopez-Trascasa M: Functional analysis in serum from atypical hemolytic uremic syndrome patients reveals impaired protection of host cells associated with mutations in factor H. *Mol Immunol* 41: 81–84, 2004
126. Taylor CM: Complement factor H and the haemolytic uraemic syndrome. *Lancet* 358: 1200–1202, 2001
127. Noris M, Brioschi S, Caprioli J, Todeschini M, Bresin E, Porrati F, Gamba S, Remuzzi G: Familial haemolytic uraemic syndrome and an MCP mutation. *Lancet* 362: 1542–1547, 2003
128. Richards A, Kemp EJ, Liszewski MK, Goodship JA, Lampe AK, Decorte R, Muslumanoglu MH, Kavukcu S, Filler G, Pirson Y, Wen LS, Atkinson JP, Goodship TH: Mutations in human complement regulator, membrane cofactor protein (CD46), predispose to development of familial hemolytic uremic syndrome. *Proc Natl Acad Sci U S A* 100: 12966–12971, 2003
129. Johnstone RW, Loveland BE, McKenzie IF: Identification and quantification of complement regulator CD46 on normal human tissues. *Immunology* 79: 341–347, 1993
130. Goodship TH, Liszewski MK, Kemp EJ, Richards A, Atkinson JP: Mutations in CD46, a complement regulatory protein, predispose to atypical HUS. *Trends Mol Med* 10: 226–231, 2004
131. Barilla-LaBarca ML, Liszewski MK, Lambris JD, Hourcade D, Atkinson JP: Role of membrane cofactor protein (CD46) in regulation of C4b and C3b deposited on cells. *J Immunol* 168: 6298–6304, 2002
132. Liszewski MK, Leung M, Cui W, Subramanian VB, Parkinson J, Barlow PN, Manchester M, Atkinson JP: Dissecting sites important for complement regulatory activity in membrane cofactor protein (MCP; CD46). *J Biol Chem* 275: 37692–37701, 2000
133. Dragon-Durey M-A, Loirat C, Cloarec S, Macher M-A, Blouin J, Nivet H, Weiss L, Fridman WH, Fremeaux-Bacchi V: Anti–factor H antibodies associated with atypical hemolytic uremic syndrome. *J Am Soc Nephrol* 16: 555–563, 2005
134. Endoh M, Yamashina M, Ohi H, Funahashi K, Ikuno T, Yasugi T, Atkinson JP, Okada H: Immunohistochemical demonstration of membrane cofactor protein (MCP) of complement in normal and diseased kidney tissues. *Clin Exp Immunol* 94: 182–188, 1993
135. Ichida S, Yuzawa Y, Okada H, Yoshioka K, Matsuo S: Localization of the complement regulatory proteins in the normal human kidney. *Kidney Int* 46: 89–96, 1994
136. Nakanishi I, Moutabarrik A, Hara T, Hatanaka M, Hayashi T, Syouji T, Okada N, Kitamura E, Tsubakihara Y, Matsumoto M, *et al.*: Identification and characterization of membrane cofactor protein (CD46) in the human kidneys. *Eur J Immunol* 24: 1529–1535, 1994
137. Fremeaux-Bacchi V, Dragon-Durey MA, Blouin J, Vigneau C, Kuypers D, Boudailliez B, Loirat C, Rondeau E, Fridman WH: Complement factor I: A susceptibility gene for atypical haemolytic uraemic syndrome. *J Med Genet* 41: e84, 2004
138. Hollenbeck M, Kutkuhn B, Aul C, Leschke M, Willers R, Grabensee B: Haemolytic-uraemic syndrome and thrombotic-thrombocytopenic purpura in adults: Clinical findings and prognostic factors for death and end-stage renal disease. *Nephrol Dial Transplant* 13: 76–81, 1998
139. Lara PN Jr, Coe TL, Zhou H, Fernando L, Holland PV, Wun T: Improved survival with plasma exchange in patients with thrombotic thrombocytopenic purpura-hemolytic uremic syndrome. *Am J Med* 107: 573–579, 1999
140. Morel-Maroger L, Kanfer A, Solez K, Sraer JD, Richet G: Prognostic importance of vascular lesions in acute renal failure with microangiopathic hemolytic anemia (hemolytic-uremic syndrome): Clinicopathologic study in 20 adults. *Kidney Int* 15: 548–558, 1979
141. Dundas S, Murphy J, Soutar RL, Jones GA, Hutchinson SJ, Todd WT: Effectiveness of therapeutic plasma exchange in the 1996 Lanarkshire *Escherichia coli* O157:H7 outbreak. *Lancet* 354: 1327–1330, 1999
142. Tsai HM: Is severe deficiency of ADAMTS-13 specific for thrombotic thrombocytopenic purpura? Yes. *J Thromb Haemost* 1: 625–631, 2003
143. Tsai HM: Deficiency of ADAMTS-13 in thrombotic and thrombocytopenic purpura. *J Thromb Haemost* 1: 2038–2040; discussion 2040–2035, 2003
144. Remuzzi G: Is ADAMTS-13 deficiency specific for thrombotic thrombocytopenic purpura? No. *J Thromb Haemost* 1: 632–634, 2003
145. Clark WF, Rock GA, Buskard N, Shumak KH, LeBlond P, Anderson D, Sutton DM: Therapeutic plasma exchange: An update from the Canadian Apheresis Group. *Ann Intern Med* 131: 453–462, 1999
146. Vesely SK, George JN, Lammle B, Studt JD, Alberio L, El-Harake MA, Raskob GE: ADAMTS13 activity in thrombotic thrombocytopenic purpura-hemolytic uremic syndrome: Relation to presenting features and clinical outcomes in a prospective cohort of 142 patients. *Blood* 102: 60–68, 2003
147. Tostivint I, Mougenot B, Flahault A, Vigneau C, Costa MA, Haymann JP, Sraer JD, Rondeau E: Adult haemolytic and uraemic syndrome: Causes and prognostic factors in the last decade. *Nephrol Dial Transplant* 17: 1228–1234, 2002
148. Ruggenenti P, Galbusera M, Cornejo RP, Bellavita P, Remuzzi G: Thrombotic thrombocytopenic purpura: Evidence that infusion rather than removal of plasma induces remission of the disease. *Am J Kidney Dis* 21: 314–318, 1993
149. Allford SL, Hunt BJ, Rose P, Machin SJ: Guidelines on the diagnosis and management of the thrombotic microangiopathic haemolytic anaemias. *Br J Haematol* 120: 556–573, 2003
150. Gerber A, Kirchhoff-Moradpour AH, Obieglo S, Brandis M, Kirschfink M, Zipfel PF, Goodship JA, Zimmerhackl LB: Successful (?) therapy of hemolytic-uremic syndrome

with factor H abnormality. *Pediatr Nephrol* 18: 952–955, 2003
151. Nathanson S, Fremeaux-Bacchi V, Deschenes G: Successful plasma therapy in hemolytic uremic syndrome with factor H deficiency. *Pediatr Nephrol* 16: 554–556, 2001
152. Stratton JD, Warwicker P: Successful treatment of factor H-related haemolytic uraemic syndrome. *Nephrol Dial Transplant* 17: 684–685, 2002
153. Remuzzi G, Galbusera M, Salvadori M, Rizzoni G, Paris S, Ruggenenti P: Bilateral nephrectomy stopped disease progression in plasma-resistant hemolytic uremic syndrome with neurological signs and coma. *Kidney Int* 49: 282–286, 1996
154. Franco A, Hernandez D, Capdevilla L, Errasti P, Gonzalez M, Ruiz JC, Sanchez J: De novo hemolytic-uremic syndrome/thrombotic microangiopathy in renal transplant patients receiving calcineurin inhibitors: Role of sirolimus. *Transplant Proc* 35: 1764–1766, 2003
155. Miller RB, Burke BA, Schmidt WJ, Gillingham KJ, Matas AJ, Mauer M, Kashtan CE: Recurrence of haemolytic-uraemic syndrome in renal transplants: A single-centre report. *Nephrol Dial Transplant* 12: 1425–1430, 1997
156. Donne RL, Abbs I, Barany P, Elinder CG, Little M, Conlon P, Goodship TH: Recurrence of hemolytic uremic syndrome after live related renal transplantation associated with subsequent de novo disease in the donor. *Am J Kidney Dis* 40: E22, 2002
157. Remuzzi G, Ruggenenti P, Codazzi D, Noris M, Caprioli J, Locatelli G, Gridelli B: Combined kidney and liver transplantation for familial haemolytic uraemic syndrome. *Lancet* 359: 1671–1672, 2002
158. Remuzzi G, Ruggenenti P, Gridelli B, Bertani A, Bettinaglio P, Bucchioni S, Sonzogni A, Bonanomi E, Sonzogni V, Platt J, Perico N, Noris M: Hemolytic uremic syndrome: A fatal outcome after kidney and liver transplantation performed to correct factor H gene mutation. *Am J Transpl* 2005, in press
159. MacConnachie AA, Todd WA: Potential therapeutic agents for the prevention and treatment of haemolytic uraemic syndrome in shiga toxin producing *Escherichia coli* infection. *Curr Opin Infect Dis* 17: 479–482, 2004
160. Pinyon RA, Paton JC, Paton AW, Botten JA, Morona R: Refinement of a therapeutic Shiga toxin-binding probiotic for human trials. *J Infect Dis* 189: 1547–1555, 2004
161. Paton AW, Morona R, Paton JC: A new biological agent for treatment of Shiga toxigenic *Escherichia coli* infections and dysentery in humans. *Nat Med* 6: 265–270, 2000
162. Takahashi M, Taguchi H, Yamaguchi H, Osaki T, Komatsu A, Kamiya S: The effect of probiotic treatment with Clostridium butyricum on enterohemorrhagic *Escherichia coli* O157:H7 infection in mice. *FEMS Immunol Med Microbiol* 41: 219–226, 2004
163. Mulvey GL, Marcato P, Kitov PI, Sadowska J, Bundle DR, Armstrong GD: Assessment in mice of the therapeutic potential of tailored, multivalent Shiga toxin carbohydrate ligands. *J Infect Dis* 187: 640–649, 2003
164. Matise I, Cornick NA, Booher SL, Samuel JE, Bosworth BT, Moon HW: Intervention with Shiga toxin (Stx) antibody after infection by Stx-producing *Escherichia coli*. *J Infect Dis* 183: 347–350, 2001
165. Raife T, Friedman KD, Fenwick B: Lepirudin prevents lethal effects of Shiga toxin in a canine model. *Thromb Haemost* 92: 387–393, 2004
166. Kirschfink M: Targeting complement in therapy. *Immunol Rev* 180: 177–189, 2001
167. Hillmen P, Hall C, Marsh JC, Elebute M, Bombara MP, Petro BE, Cullen MJ, Richards SJ, Rollins SA, Mojcik CF, Rother RP: Effect of eculizumab on hemolysis and transfusion requirements in patients with paroxysmal nocturnal hemoglobinuria. *N Engl J Med* 350: 552–559, 2004
168. Granger CB, Mahaffey KW, Weaver WD, Theroux P, Hochman JS, Filloon TG, Rollins S, Todaro TG, Nicolau JC, Ruzyllo W, Armstrong PW: Pexelizumab, an anti-C5 complement antibody, as adjunctive therapy to primary percutaneous coronary intervention in acute myocardial infarction: The Complement Inhibition in Myocardial Infarction Treated with Angioplasty (COMMA) trial. *Circulation* 108: 1184–1190, 2003
169. Li JS, Sanders SP, Perry AE, Stinnett SS, Jaggers J, Bokesch P, Reynolds L, Nassar R, Anderson PA: Pharmacokinetics and safety of TP10, soluble complement receptor 1, in infants undergoing cardiopulmonary bypass. *Am Heart J* 147: 173–180, 2004
170. Lazar HL, Bokesch PM, van Lenta F, Fitzgerald C, Emmett C, Marsh HC Jr, Ryan U: Soluble human complement receptor 1 limits ischemic damage in cardiac surgery patients at high risk requiring cardiopulmonary bypass. *Circulation* 110[Suppl 1]: II274–II279, 2004

CHAPTER 8

General Discussion and Conclusions

General Discussion

Reduced serum levels of the third component (C3) of complement have been reported since 1974 in both familial and sporadic forms of non-Stx-HUS [48, 51, 63], which were taken as to reflect C3 activation and consumption in the microvasculature [69, 82]. However epidemiological evidence of an association between complement activation and non-Stx-HUS was lacking. In **chapter 2** we evaluated serum levels of C3 of C4 in familial cases of HUS from the International Registry of HUS and TTP. Patients with the familial form of TTP were investigated as well, based on data that the two syndromes have many overlapping clinical features and markers of C3 activation were also reported in cases with TTP [83, 84].

Reduced C3 levels were found in around 70% of cases irrespective on whether they had a clinical diagnosis of HUS or TTP [51]. Of note serum C3 levels were depressed even during remission of the disease, which excludes that low C3 levels could derive from consumption in a still-ongoing microangiopathic process, and rather indicates a congenital defect leading to chronic complement activation. In support of this possibility, low C3 levels were also found in around 25% of the relatives of our patients who had never had signs of the disease, which confirms clustering of the defect within families. At multivariate analysis C3 serum levels were significantly associated with HUS and TTP within affected families and in the overall study population and subjects with decreased C3 had a relative risk of HUS and TTP of more than 16 within families and more than 27 in the general population.

Either in cases or in case-relatives depressed C3 values were not paralleled by similar

changes in C4 levels. The latter finding indicates that hypocomplementemia in our series was caused by a selective activation of the alternative pathway of complement that causes the cleavage of C3 but not of C4, at variance with activation of the classical pathway that is associated with both C3 and C4 consumption.

In previous studies, in occasional families including one or more cases of HUS, low C3 levels were accompanied by very low levels of CFH, a regulatory protein that inhibits complement activation through the alternative pathway [65, 73]. The above data raised the possibility that low C3 in the setting of familial HUS may depend on an uncontrolled activation of the alternative pathway of complement secondary to an inherited deficiency of CFH. In support to this possibility, in studies of **chapter 2** we found CFH abnormalities (i.e. either very low serum CFH levels or abnormal mobility of CFH on Western blot) in four cases within two different families but in none of the healthy unrelated controls [47]. In addition, within families CFH abnormalities correlated with C3 reduction. These results are in line with the evidence provided by Warwicker and coworkers [49] in three large families with HUS, that an area on chromosome 1q, where CFH is mapped, segregates with the disease. Affected members and obligate carriers within one family were found, by mutation analysis, to have heterozygous point mutations in *CFH* gene. In studies of **chapters 3 and 4** mutation screening was undertaken in a large series of patients with non-Stx-HUS from the International Registry of HUS and TTP, to establish whether mutations in *CFH* were actually involved in determining predisposition to familial forms of non-Stx-HUS and to evaluate whether this could apply to sporadic forms of the disease as well.

In these studies 17 independent *CFH* mutational events were found in 33 out of 101 patients with non-Stx-HUS [52, 85]. The frequency of *CFH* mutations was high in familial cases (46%), but rather low (17%) in sporadic cases. All the *CFH* mutations found in non-Stx-HUS patients are heterozygous mutations, with the exception of one homozygous mutation found in a Bedouin family with recessive transmission. Of note, most of them cause single amino acid change, while three mutations introduced premature stop codons. Although the identified mutations are spread over several exons, 70% of them clustered in exons XXII and XXIII and affected the most C terminus part of CFH (SCR20). Heterozygous mutations were associated with normal CFH levels in most cases , whereas the homozygous mutation in the Bedouin family resulted in severe reduction of CFH levels accompanied by reduced RNA expression [52, 85].

The above results have been confirmed by other groups. Thus, more than 50 different *CFH* mutations have been identified till now in 80 patients who had familial and sporadic forms of non-Stx-HUS [49, 52, 56, 85-89]. Forty-eight out of 50 *CFH* mutations are heterozygous and mainly cluster in the C-terminus domains and are commonly associated with normal CFH plasma levels. This is at variance with patients with type II membranoproliferative glomerulonephritis who carry homozygous *CFH* mutations causing severely reduced CFH levels [87]. Expression and functional studies done by our group in collaboration with Peter Zipfel ([79] and **chapter 7**) and by other groups [90, 91] demonstrated that CFH proteins carrying HUS-associated mutations have a severely reduced capability to interact with polyanions and with surface-bound C3b [79, 90, 91],

which results in a lower density of mutant CFH molecules bound to endothelial cells surface and in a diminished complement regulatory activity on the cell membrane [79, 91]. In contrast these mutants have a normal capacity to control activation of the complement in plasma, as indicated by data that they retain a normal cofactor activity in the proteolysis of fluid-phase C3b [90]. The latter finding explains the case of patients with HUS and *CFH* mutations who have normal or slightly reduced serum C3 levels [86, 87].

Analysis of clinical data from patients included in **chapter 4** [52] showed that the disease manifested earlier and was associated with a higher mortality rate in carriers of *CFH* mutations than in those without *CFH* mutations. However, a consistent number of patients carrying heterozygous *CFH* mutations had long remissions and presented late in life. By contrast the only homozygous *CFH* mutation in our series was associated with a neonatal onset and a very high mortality rate.

Patients carrying heterozygous *CFH* mutations have a partial CFH deficiency, as a result of one intact and one defective allele, which more likely predispose to, rather than directly cause the disease. Thus, a "second hit" is needed to fully manifestation of the disease, as also documented by the incomplete (50%) penetrance of the *CFH* mutations found in our families. Actually, conditions that trigger complement activation, either directly (bacterial and viral infections) or indirectly, by causing endothelial insult (drugs, systemic diseases, pregnancy), were found to precipitate the acute event in around 60% of patients with *CFH* mutations reported in **chapters 3 and 4** [52, 85]. All the above observations can be reconciled by reasoning that in these patients the suboptimal CFH activity is enough to protect the host

from complement activation in physiological conditions. However, upon exposure to an agent that activates complement, C3b is formed in higher than normal amounts and its deposition on vascular endothelial cells can not be fully prevented due to loss of polyanion binding capability of mutated CFH. This abnormality favors the formation of MAC and the recruitment of inflammatory cells, all events causing damage and retraction of endothelial cells, adhesion and aggregation of platelets. The glomerular capillary bed is a fenestrated endothelium and the exposed basement membrane supplies a surface that is rich in polyanions for CFH binding, an event that is crucial to prevent C3b deposition and complement activation since subendothelial matrix lacks endogenous complement regulators. The latter observation could explain the renal localization of microvascular injury of HUS.

Two third of patients with non-Stx-HUS in our series had no *CFH* mutations, despite up to 50% of them exhibit evidence of overactivity of the alternative pathway of complement [52]. The possibility that uncommon polymorphic variants of *CFH* gene may confer susceptibility to HUS in subjects without *CFH* mutations has been investigated in studies of **chapter 4**. It was found that the T allele of the C-257T, the G allele of the A2089G and the T allele of the G2881T polymorphisms of *CFH* gene were more frequent in HUS patients without *CFH* mutations than in healthy subjects [52] and analysis of the overall study population revealed that subjects carrying two or three of the above variants had a four-fold increased risk of developing HUS [52]. We also found that the 257T, 2089G and 2881T alleles might also have a role in determining the penetrance [52] of the disease in *CFH* mutation

carriers. In 5 out of 9 families, subjects who developed HUS had inherited an allele carrying the *CFH* mutation from one parent together with an allele carrying at least one disease-associated *CFH* polymorphism from the other parent. Instead all the healthy *CFH* mutation carriers only inherited the mutation but no polymorphism [52].

Much debate has been emerging in the recent years on whether it was possible or not to make a differential diagnosis between HUS and TTP [92, 93]. The two syndromes have in common microangiopathic hemolytic anemia, consumption thrombocytopenia and microvascular thrombosis, but differ because of severe renal involvement, often with renal failure in HUS, and prevalent central nervous system symptoms in TTP [1]. In studies of **chapter 4**, we investigated whether *CFH* gene mutations and polymorphisms may have a role in determining predisposition to TTP. We found that none of the 32 patients with a diagnosis of TTP and no renal impairment studied in **chapter 4** carried *CFH* mutations, which indicates that CFH abnormalities are specifically associated with renal manifestations of the thrombotic microangiopathy. It is now known that 70 to 80% of case of TTP are triggered by a deficiency of ADAMTS13 [75], a plasma metalloprotease that cleaves von Willebrand factor multimers soon after their secretion by either endothelial cells or megakaryocytes. Two primary mechanisms for deficiency of ADAMTS13 activity have been identified, namely a constitutive deficiency, caused by compound heterozygous or homozygous *ADAMTS13* mutations, and an acquired deficiency caused by the presence of inhibitory antibodies [74]. The observation that in large clinical studies [75, 76] ADAMTS13 activity was defective in patients with diagnosis of TTP but not in those with HUS generated

the paradigm that TTP is due to a deficiency of ADAMTS13, in turn not involved in the pathogenesis of HUS. However rare cases with ADAMTS13 deficiency manifest severe renal involvement, often with ESRD [77, 78]. Those patients' clinical manifestations are difficult do distinguish from those of HUS. In studies of **chapter 5** we investigated the genetic basis of phenotype heterogeneity in patients with ADAMTS13 deficiency. We studied a family with two affected sisters, one who presented with exclusive neurological symptoms and the other with severe renal involvement that required chronic dialysis [94]. The two patients had complete ADAMTS13 deficiency as a result of two heterozygous mutations. In addition, a heterozygous mutation in *CFH* gene was found in the patient who developed chronic renal failure but not in her sister with only neurological symptoms. Based on these data we hypothesize that in the former patient CFH haploinsufficency had a role in determining the renal complications that superimposed on the systemic disease caused by ADAMTS13 deficiency.

In studies of **chapter 6** we searched for additional genes whose alterations may predispose to non-Stx-HUS. To this purpose, 25 patients with familial and sporadic forms of non-Stx-HUS with no *CFH* mutation were screened for mutations in genes encoding for complement regulatory proteins. The attention was focused on genes of the RCA cluster, encoding factor H related 5 (FHR5), complement receptor 1 (CR1) and membrane cofactor protein (MCP), based on Warwicker's data [49] showing that two of the original three families whose defect mapped to the RCA cluster did not have a *CFH* mutation. Results showed no disease-associated mutation in *FHR5* nor in *CR1*. By contrast, we found a

heterozygous mutation in *MCP*, encoding a cell-bound membrane complement regulator, in two siblings with a history of HUS [95]. MCP is a widely expressed transmembrane glycoprotein that serves as a cofactor for factor I to cleave C3b and C4b deposited on host cell surface [59, 96, 97]. The mutation causes three amino acid changes and a premature interruption of MCP protein in SCR4, which result in 50% reduction in MCP expression levels on PBMC of heterozygous individuals. These results have been confirmed by another group who found a heterozygous deletion of D237/S238 aminoacids in one family and a S206P substitution in two families [98]. As reported in **chapter 7**, more recent studies from our group on 112 patients with non-Stx-HUS have revealed five additional *MCP* mutations in familial (7 cases) and in sporadic (5 cases) HUS with a mutation frequency of 11% (25 % in familial and 6% in sporadic forms) (abstract; Brioschi et al. VII Congress of the Italian society of Human Genetics, 13-15 October 2004).

MCP is highly expressed in the kidney [60-62]. It likely exerts a main role in protecting glomerular endothelial cells against C3 activation as indicated by data that cofactor activity in the extracts of these cells was completely blocked by anti-MCP antibody [61]. As hypothesized for *CFH*, mutations in *MCP* likely predispose rather than directly cause HUS. Upon exposure to conditions that cause activation of the complement cascade, reduced levels or defective C3b binding capability and cofactor activity of mutated MCP on glomerular endothelial cells would result in an insufficient protection of these cells from complement activation.

These findings have potential clinical implications. Genotyping of patients with

non-Stx-HUS could potentially allow to optimize treatment. Plasma infusion or exchange has been used in published case reports to treat patients with *CFH* mutations [82, 99-101]. The rationale is to provide patients with normal CFH to correct their genetic deficiency, but the efficacy of this approach remains questionable. Results from studies of **chapter 4** and unpublished results from the International Registry of HUS and TTP show that around 50% of patients with *CFH* mutations achieved either complete or partial remission (hematological normalization with renal sequelae) after receiving plasma; however, the remaining 50% did not respond and 10-20% died during the acute episode. Unlike CFH, MCP is a transmembrane protein, so the rationale for using plasma in patients with MCP mutations is less clear. Data from **chapter 6** and unpublished results from our Registry indicate that 70-80% of patients achieve remission following plasma infusion or exchange; however complete recovery from the acute episode was also observed in 70-80% of patients not treated with plasma.

It is debatable whether kidney transplantation is an appropriate treatment for non-Stx-HUS patients who have progressed to ESRD. In published studies [102, 103], around 50% of non-Stx-HUS patients given a renal transplant had a recurrence of the disease in the grafted organ. There is no effective treatment for recurrence and graft failure occurs in more than 90% of patients who experience disease recurrence. In **chapter 4 and 7** we evaluated whether the specific genetic defect underlying non-Stx-HUS influences the outcome of the kidney graft. We found that in patients with *CFH* mutations the graft outcome is poor and the recurrence rate on the transplanted organ is significantly higher

than in patients without *CFH* mutations [52, 94]}. As CFH is a plasma protein mainly produced by the liver, the genetic defect was not corrected by the kidney transplant and persistent CFH deficiency predisposed to disease recurrence on the graft. As reported in **chapter 7** [94], simultaneous kidney and liver transplant has been recently performed by our group in two young children with non-SLT-HUS and *CFH* mutations with the rationale to correct the genetic defect and prevent disease recurrence; however, both cases were complicated by irreversible liver failure [80, 81]. The first case recovered after a second uneventful liver transplantation. The child, who used to have monthly recurrences before transplantation, has had no symptoms of HUS for more than 2-years follow-up. The second case had a fatal, primary non-function of the liver graft followed by multi-organ failure and patient's death. The reasons for this may include increase susceptibility of the transplanted liver to ischemic or immune injury related to uncontrolled complement activation. Thus, despite the potential to correct the genetic defect, combined kidney and liver transplant for non-Stx-HUS associated with *CFH* mutations, should not be performed unless a patient is at imminent risk with life-threatening complications.

By contrast, patients with aberrant forms of MCP, a transmembrane protein highly expressed in the kidney, have favorable kidney graft outcomes, as shown by data from four patients (**chapter 7**) who were successfully transplanted with no disease recurrence [94, 98]. These preliminary data suggest that genotyping for *CFH* and *MCP* should be performed in all patients with ESRD secondary to non-Stx-HUS who are candidates for transplantation, to help define graft prognosis.

Conclusion

The results of this thesis, in particular the discovery of mutations in two different complement regulatory genes, provide enough evidence of the involvement of complement activation in the pathogenesis of non-Stx-HUS. This concept has been recently reinforced by studies from other authors, showing mutations in the gene encoding factor I (*IF*), a serine protease that cleaves the alpha chian of C3b, in five sporadic cases (**chapter 7** and [104, 105]).

Complement regulatory proteins are essential to prevent non-specific damage of host endothelial cells and limit deposition of C3b to the surface of pathogens [53, 54]. Thus, in patients with genetic alterations causing defective activity of complement regulatory proteins, exposure to an agent that activates complement results in C3b deposition on vascular endothelial cells and on subendothelial matrix and in the formation of the C3 and C5 convertase complexes. The proteolysis of C3 and C5 by convertases causes the release of chemotactic anaphylatoxins (C3a and C5a) that recruit inflammatory cells toward the endothelial layer. On the other hand, the deposition of C3b is followed by the formation of MAC, which leads to endothelial cells injury and detachment and to sublytic perturbation, leading to endothelial activation and expression of adhesion molecules. The latter favors leukocyte attachment and activation with the release of oxygen radicals and proteinases that further damage the endothelium. After endothelial damage, cell detachment ensues and exposes basement membranes. In these conditions, platelets from the circulation adhere and aggregate to the exposed matrix.

Hopefully, these findings will translate into an improvement in the management of patients with this disease and will pave the way for tailored treatments. Complement inhibitors, such as the humanized monoclonal antibodies directed against C5 and the soluble forms of the C3/C5 convertase inhibitor CR1, are currently in clinical and preclinical development [106]. These drugs should be useful in blocking complement-mediated kidney damage during the acute episode of HUS or in preventing recurrence after kidney transplantation. Complement inhibitors could also be theoretically of benefit to prevent complications, such as primary liver non-function, in combined kidney and liver transplantation in patients with *CFH* genetic defects.

Specific replacement therapy with recombinant or purified plasma proteins could become a viable alternative to plasma in patients with *CFH* or *IF* mutations.

References

1. Ruggenenti P, Noris M, Remuzzi G: Thrombotic microangiopathy, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura. *Kidney Int* 60:831-846, 2001
2. Kaplan BS, Meyers KE, Schulman SL: The pathogenesis and treatment of hemolytic uremic syndrome. *J Am Soc Nephrol* 9:1126-1133, 1998
3. Taylor CM, Howie AJ, Williams JM: No common final pathogenetic pathway in haemolytic uraemic syndromes. *Nephrol Dial Transplant* 14:1100-1102, 1999
4. Remuzzi G, Ruggenenti P: Thrombotic microangiopathies, in *Renal Pathology*, edited by Tisher C, Brenner B, second ed, Philadelphia, J.B. Lippincott, 1994, pp 1154-1184
5. Kincaid-Smith P: Participation of intravascular coagulation in the pathogenesis of glomerular and vascular lesions. *Kidney Int* 7:242-253, 1975
6. Levy M, Gagnadoux M, Habib R: Pathology of hemolytic uremic syndrome in children, in *Hemostasis, prostaglandins and renal disease*, edited by Remuzzi G, Mecca G, G dG, New York, Raven Press, 1980, pp 383-397
7. Vitsky BH, Suzuki Y, Strauss L, Churg J: The hemolytic-uremic syndrome: a study of renal pathologic alterations. *Am J Pathol* 57:627-647, 1969
8. Proia AD, Harden EA, Silberman HR: Mitomycin-induced hemolytic-uremic syndrome. *Arch Pathol Lab Med* 108:959-962, 1984
9. Thorpe CM: Shiga toxin-producing Escherichia coli infection. *Clin Infect Dis* 38:1298-1303, 2004
10. Karmali MA, Steele BT, Petric M, Lim C: Sporadic cases of haemolytic-uraemic syndrome associated with faecal cytotoxin and cytotoxin-producing Escherichia coli in stools. *Lancet* 1:619-620, 1983
11. Riley LW, Remis RS, Helgerson SD, McGee HB, Wells JG, Davis BR, Hebert RJ, Olcott ES, Johnson LM, Hargrett NT, Blake PA, Cohen ML: Hemorrhagic colitis associated with a rare Escherichia coli serotype. *N Engl J Med* 308:681-685, 1983
12. Mead PS, Griffin PM: Escherichia coli O157:H7. *Lancet* 352:1207-1212, 1998
13. Jackson MP, Newland JW, Holmes RK, O'Brien AD: Nucleotide sequence analysis of the structural genes for Shiga-like toxin I encoded by bacteriophage 933J from Escherichia coli. *Microb Pathog* 2:147-153, 1987
14. Brooks JT, Bergmire-Sweat D, Kennedy M, Hendricks K, Garcia M, Marengo L, Wells J, Ying M, Bibb W, Griffin PM, Hoekstra RM, Friedman CR: Outbreak of Shiga toxin-producing Escherichia coli O111:H8 infections among attendees of a high school cheerleading camp. *Clin Infect Dis* 38:190-198, 2004
15. Varma JK, Greene KD, Reller ME, DeLong SM, Trottier J, Nowicki SF, DiOrio M, Koch EM, Bannerman TL, York ST, Lambert-Fair MA, Wells JG, Mead PS: An outbreak of Escherichia coli O157 infection following

exposure to a contaminated building. *Jama* 290:2709-2712, 2003

16. Caprioli A, Luzzi I, Rosmini F, Pasquini P, Cirrincione R, Gianviti A, Matteucci MC, Rizzoni G: Hemolytic-uremic syndrome and Vero cytotoxin-producing Escherichia coli infection in Italy. The HUS Italian Study Group. *J Infect Dis* 166:154-158, 1992

17. Ludwig K, Bitzan M, Zimmermann S, Kloth M, Ruder H, Muller-Wiefel DE: Immune response to non-O157 Vero toxin-producing Escherichia coli in patients with hemolytic uremic syndrome. *J Infect Dis* 174:1028-1039, 1996

18. McCarthy TA, Barrett NL, Hadler JL, Salsbury B, Howard RT, Dingman DW, Brinkman CD, Bibb WF, Cartter ML: Hemolytic-Uremic Syndrome and Escherichia coli O121 at a Lake in Connecticut, 1999. *Pediatrics* 108:E59, 2001

19. Sonntag AK, Prager R, Bielaszewska M, Zhang W, Fruth A, Tschape H, Karch H: Phenotypic and genotypic analyses of enterohemorrhagic Escherichia coli O145 strains from patients in Germany. *J Clin Microbiol* 42:954-962, 2004

20. Lopez EL, Diaz M, Grinstein S, Devoto S, Mendilaharzu F, Murray BE, Ashkenazi S, Rubeglio E, Woloj M, Vasquez M, et al.: Hemolytic uremic syndrome and diarrhea in Argentine children: the role of Shiga-like toxins. *J Infect Dis* 160:469-475, 1989

21. Gianviti A, Rosmini F, Caprioli A, Corona R, Matteucci MC, Principato F, Luzzi I, Rizzoni G: Haemolytic-uraemic syndrome in childhood: surveillance and case-control studies in Italy. Italian HUS Study Group. *Pediatr Nephrol* 8:705-709, 1994

22. Tonshoff B, Sammet A, Sanden I, Mehls O, Waldherr R, Scharer K: Outcome and prognostic determinants in the hemolytic uremic syndrome of children. *Nephron* 68:63-70, 1994

23. Banatvala N, Griffin PM, Greene KD, Barrett TJ, Bibb WF, Green JH, Wells JG: The United States National Prospective Hemolytic Uremic Syndrome Study: microbiologic, serologic, clinical, and epidemiologic findings. *J Infect Dis* 183:1063-1070, 2001

24. Milford D: The hemolytic uremic syndromes in the United Kingdom, in *Hemolytic uremic syndrome and thrombotic thrombocytopenic purpura*, edited by Kaplan BS TR, Moake JL, New York, Marcel Dekker Inc., 1992, pp 39-59

25. Van Dyck M, Proesmans W, Depraetere M: Hemolytic uremic syndrome in childhood: renal function ten years later. *Clin Nephrol* 29:109-112, 1988

26. Locking ME, O'Brien SJ, Reilly WJ, Wright EM, Campbell DM, Coia JE, Browning LM, Ramsay CN: Risk factors for sporadic cases of Escherichia coli O157 infection: the importance of contact with animal excreta. *Epidemiol Infect* 127:215-220, 2001

27. Mead PS, Finelli L, Lambert-Fair MA, Champ D, Townes J, Hutwagner L, Barrett T, Spitalny K, Mintz E: Risk

factors for sporadic infection with Escherichia coli O157:H7. *Arch Intern Med* 157:204-208, 1997

28. Chandler WL, Jelacic S, Boster DR, Ciol MA, Williams GD, Watkins SL, Igarashi T, Tarr PI: Prothrombotic coagulation abnormalities preceding the hemolytic-uremic syndrome. *N Engl J Med* 346:23-32, 2002

29. Beatty ME, Griffin PM, Tulu AN, Olsen SJ: Culturing practices and antibiotic use in children with diarrhea. *Pediatrics* 113:628-629, 2004

30. Garg AX, Suri RS, Barrowman N, Rehman F, Matsell D, Rosas-Arellano MP, Salvadori M, Haynes RB, Clark WF: Long-term renal prognosis of diarrhea-associated hemolytic uremic syndrome: a systematic review, meta-analysis, and meta-regression. *Jama* 290:1360-1370, 2003

31. Schieppati A, Ruggenenti P, Cornejo RP, Ferrario F, Gregorini G, Zucchelli P, Rossi E, Remuzzi G: Renal function at hospital admission as a prognostic factor in adult hemolytic uremic syndrome. The Italian Registry of Haemolytic Uremic Syndrome. *J Am Soc Nephrol* 2:1640-1644, 1992

32. Taylor CM, Chua C, Howie AJ, Risdon RA: Clinico-pathological findings in diarrhoea-negative haemolytic uraemic syndrome. *Pediatr Nephrol* 19:419-425, 2004

33. Constantinescu AR, Bitzan M, Weiss LS, Christen E, Kaplan BS, Cnaan A, Trachtman H: Non-enteropathic hemolytic uremic syndrome: causes and short-term course. *Am J Kidney Dis* 43:976-982, 2004

34. Hall SM, Glickman M: The British Paediatric Surveillance Unit. *Arch Dis Child* 63:344-346, 1988

35. Renaud C, Niaudet P, Gagnadoux MF, Broyer M, Habib R: Haemolytic uraemic syndrome: prognostic factors in children over 3 years of age. *Pediatr Nephrol* 9:24-29, 1995

36. Erickson LC, Smith WS, Biswas AK, Camarca MA, Waecker NJ, Jr.: Streptococcus pneumoniae-induced hemolytic uremic syndrome: a case for early diagnosis. *Pediatr Nephrol* 8:211-213, 1994

37. Novak RW, Martin CR, Orsini EN: Hemolytic-uremic syndrome and T-cryptantigen exposure by neuraminidase-producing pneumococci: an emerging problem? *Pediatr Pathol* 1:409-413, 1983

38. Leaf AN, Laubenstein LJ, Raphael B, Hochster H, Baez L, Karpatkin S: Thrombotic thrombocytopenic purpura associated with human immunodeficiency virus type 1 (HIV-1) infection. *Ann Intern Med* 109:194-197, 1988

39. Dlott JS, Danielson CF, Blue-Hnidy DE, McCarthy LJ: Drug-induced thrombotic thrombocytopenic purpura/hemolytic uremic syndrome: a concise review. *Therap Apher Dial* 8:102-111, 2004

40. Reynolds JC, Agodoa LY, Yuan CM, Abbott KC: Thrombotic microangiopathy after renal transplantation in the United States. *Am J Kidney Dis* 42:1058-1068, 2003

41. Ruggenenti P: Post-transplant hemolytic-uremic syndrome. *Kidney Int* 62:1093-1104, 2002

42. Remuzzi G, Ruggenenti P: The hemolytic uremic syndrome. *Kidney Int* 48:2-19, 1995

43. George JN: The association of pregnancy with thrombotic thrombocytopenic purpura-hemolytic uremic syndrome. *Curr Opin Hematol* 10:339-344, 2003

44. Campbell S, Carre IJ: Fatal haemolytic uraemic syndrome and idiopathic hyperlipaemia in monozygotic twins. *Arch Dis Child* 40:654-658, 1965

45. Kaplan BS, Chesney RW, Drummond KN: Hemolytic uremic syndrome in families. *N Engl J Med* 292:1090-1093, 1975

46. Berns JS, Kaplan BS, Mackow RC, Hefter LG: Inherited hemolytic uremic syndrome in adults. *Am J Kidney Dis* 19:331-334, 1992

47. Noris M, Azzollini N, Mister M, Pezzotta A, Piccinini G, Casiraghi F, Cugini D, Perico N, Orisio S, Remuzzi G: Peripheral donor leukocytes prolong survival of rat renal allografts. *Kidney Int* 56:1101-1112, 1999

48. Carreras L, Romero R, Requesens C, Oliver AJ, Carrera M, Clavo M, Alsina J: Familial hypocomplementemic hemolytic uremic syndrome with HLA-A3,B7 haplotype. *Jama* 245:602-604, 1981

49. Warwicker P, Goodship TH, Donne RL, Pirson Y, Nicholls A, Ward RM, Turnpenny P, Goodship JA: Genetic studies into inherited and sporadic hemolytic uremic syndrome. *Kidney Int* 53:836-844, 1998

50. Petermann A, Offermann G, Distler A, Sharma AM: Familial hemolytic-uremic syndrome in three generations. *Am J Kidney Dis* 32:1063-1067, 1998

51. Noris M, Ruggenenti P, Perna A, Orisio S, Caprioli J, Skerka C, Vasile B, Zipfel PF, Remuzzi G: Hypocomplementemia discloses genetic predisposition to hemolytic uremic syndrome and thrombotic thrombocytopenic purpura: role of factor H abnormalities. Italian Registry of Familial and Recurrent Hemolytic Uremic Syndrome/Thrombotic Thrombocytopenic Purpura. *J Am Soc Nephrol* 10:281-293, 1999

52. Caprioli J, Castelletti F, Bucchioni S, Bettinaglio P, Bresin E, Pianetti G, Gamba S, Brioschi S, Daina E, Remuzzi G, Noris M: Complement factor H mutations and gene polymorphisms in haemolytic uraemic syndrome: the C-257T, the A2089G and the G2881T polymorphisms are strongly associated with the disease. *Hum Mol Genet* 12:3385-3395, 2003

53. Walport MJ: Complement. First of two parts. *N Engl J Med* 344:1058-1066, 2001

54. Walport MJ: Complement. Second of two parts. *N Engl J Med* 344:1140-1144, 2001

55. Zipfel PF, Skerka C: Complement factor H and related proteins: an expanding family of complement-regulatory proteins? *Immunol Today* 15:121-126, 1994

56. Rodriguez de Cordoba S, Esparza-Gordillo J, Goicoechea de Jorge E, Lopez-Trascasa M, Sanchez-Corral P: The human complement factor H: functional roles, genetic variations and disease associations. *Mol Immunol* 41:355-367, 2004

57. Pangburn MK: Cutting edge: localization of the host recognition functions of complement factor H at the carboxyl-terminal: implications for hemolytic uremic syndrome. *J Immunol* 169:4702-4706, 2002

58. Hellwage J, Jokiranta TS, Friese MA, Wolk TU, Kampen E, Zipfel PF, Meri S: Complement C3b/C3d and cell

surface polyanions are recognized by overlapping binding sites on the most carboxyl-terminal domain of complement factor H. *J Immunol* 169:6935-6944, 2002

59. Goodship TH, Liszewski MK, Kemp EJ, Richards A, Atkinson JP: Mutations in CD46, a complement regulatory protein, predispose to atypical HUS. *Trends Mol Med* 10:226-231, 2004

60. Endoh M, Yamashina M, Ohi H, Funahashi K, Ikuno T, Yasugi T, Atkinson JP, Okada H: Immunohistochemical demonstration of membrane cofactor protein (MCP) of complement in normal and diseased kidney tissues. *Clin Exp Immunol* 94:182-188, 1993

61. Nakanishi I, Moutabarrik A, Hara T, Hatanaka M, Hayashi T, Syouji T, Okada N, Kitamura E, Tsubakihara Y, Matsumoto M, et al.: Identification and characterization of membrane cofactor protein (CD46) in the human kidneys. *Eur J Immunol* 24:1529-1535, 1994

62. Ichida S, Yuzawa Y, Okada H, Yoshioka K, Matsuo S: Localization of the complement regulatory proteins in the normal human kidney. *Kidney Int* 46:89-96, 1994

63. Stuhlinger W, Kourilsky O, Kanfer A, Sraer JD: Letter: Haemolytic-uraemic syndrome: evidence for intravascular C3 activation. *Lancet* 2:788-789, 1974

64. Bogdanovic R, Cvoric A, Nikolic V, Sindjic M: Recurrent haemolytic-uraemic syndrome with hypocomplementaemia: a case report. *Pediatr Nephrol* 2:236-238, 1988

65. Pichette V, Querin S, Schurch W, Brun G, Lehner-Netsch G, Delage JM: Familial hemolytic-uremic syndrome and homozygous factor H deficiency. *Am J Kidney Dis* 24:936-941, 1994

66. Kim Y, Miller K, Michael AF: Breakdown products of C3 and factor B in hemolytic-uremic syndrome. *J Lab Clin Med* 89:845-850, 1977

67. Monnens L, Molenaar J, Lambert PH, Proesmans W, van Munster P: The complement system in hemolytic-uremic syndrome in childhood. *Clin Nephrol* 13:168-171, 1980

68. Gonzalo A, Mampaso F, Gallego N, Bellas C, Segui J, Ortuno J: Hemolytic uremic syndrome with hypocomplementemia and deposits of IgM and C3 in the involved renal tissue. *Clin Nephrol* 16:193-199, 1981

69. Hammar SP, Bloomer HA, McCloskey D: Adult hemolytic uremic syndrome with renal arteriolar deposition of IgM andC3. *Am J Clin Pathol* 70:434-439, 1978

70. Cossio PM, Laguens RP, Pantin DJ, de Bracco MM, Molinas F, Voyer LE, Arana RM: Persistent glomerulonephritis following the haemolytic-uremic syndrome. Immunopathological and morphological studies. *Clin Exp Immunol* 29:361-368, 1977

71. Barre P, Kaplan BS, de Chadarevian JP, Drummond KN: Hemolytic uremic syndrome with hypocomplementemia, serum C3NeF, and glomerular deposits of C3. *Arch Pathol Lab Med* 101:357-361, 1977

72. Ohali M, Shalev H, Schlesinger M, Katz Y, Kachko L, Carmi R, Sofer S, Landau D: Hypocomplementemic

autosomal recessive hemolytic uremic syndrome with decreased factor H. *Pediatr Nephrol* 12:619-624, 1998

73. Thompson RA, Winterborn MH: Hypocomplementaemia due to a genetic deficiency of beta 1H globulin. *Clin Exp Immunol* 46:110-119, 1981

74. Soejima K, Nakagaki T: Interplay between ADAMTS13 and von Willebrand factor in inherited and acquired thrombotic microangiopathies. *Semin Hematol* 42:56-62, 2005

75. Furlan M, Robles R, Galbusera M, Remuzzi G, Kyrle PA, Brenner B, Krause M, Scharrer I, Aumann V, Mittler U, Solenthaler M, Lammle B: von Willebrand factor-cleaving protease in thrombotic thrombocytopenic purpura and the hemolytic-uremic syndrome. *N Engl J Med* 339:1578-1584, 1998

76. Tsai HM, Lian EC: Antibodies to von Willebrand factor-cleaving protease in acute thrombotic thrombocytopenic purpura. *N Engl J Med* 339:1585-1594, 1998

77. Veyradier A, Obert B, Houllier A, Meyer D, Girma JP: Specific von Willebrand factor-cleaving protease in thrombotic microangiopathies: a study of 111 cases. *Blood* 98:1765-1772, 2001

78. Remuzzi G, Galbusera M, Noris M, Canciani MT, Daina E, Bresin E, Contaretti S, Caprioli J, Gamba S, Ruggenenti P, Perico N, Mannucci PM: von Willebrand factor cleaving protease (ADAMTS13) is deficient in recurrent and familial thrombotic thrombocytopenic purpura and hemolytic uremic syndrome. *Blood* 100:778-785, 2002

79. Manuelian T, Hellwage J, Meri S, Caprioli J, Noris M, Heinen S, Jozsi M, Neumann HP, Remuzzi G, Zipfel PF: Mutations in factor H reduce binding affinity to C3b and heparin and surface attachment to endothelial cells in hemolytic uremic syndrome. *J Clin Invest* 111:1181-1190, 2003

80. Remuzzi G, Ruggenenti P, Codazzi D, Noris M, Caprioli J, Locatelli G, Gridelli B: Combined kidney and liver transplantation for familial haemolytic uraemic syndrome. *Lancet* 359:1671-1672, 2002

81. Remuzzi G, Ruggenenti P, Colledan M, Gridelli B, Bertani A, Bettinaglio P, Bucchioni S, Sonzogni A, Bonanomi E, Sonzogni V, Platt J, Perico N, Noris M: Hemolytic uremic syndrome: a fatal outcome after kidney and liver transplantation performed to correct factor H gene mutation. *American Journal of Transplantation* 5, 2005

82. Landau D, Shalev H, Levy-Finer G, Polonsky A, Segev Y, Katchko L: Familial hemolytic uremic syndrome associated with complement factor H deficiency. *J Pediatr* 138:412-417, 2001

83. Wright JF, Wang H, Hornstein A, Hogarth M, Mody M, Garvey MB, Blanchette V, Rock G, Freedman J: Characterization of platelet glycoproteins and platelet/endothelial cell antibodies in patients with thrombotic thrombocytopenic purpura. *Br J Haematol* 107:546-555, 1999

84. Weisenburger DD, O'Conner ML, Hart MN: Thrombotic thrombocytopenic purpura with C'3 vascular deposits: report of a case. *Am J Clin Pathol* 67:61-63, 1977

85. Caprioli J, Bettinaglio P, Zipfel PF, Amadei B, Daina E, Gamba S, Skerka C, Marziliano N, Remuzzi G, Noris M:

The molecular basis of familial hemolytic uremic syndrome: mutation analysis of factor H gene reveals a hot spot in short consensus repeat 20. *J Am Soc Nephrol* 12:297-307, 2001

86. Neumann HP, Salzmann M, Bohnert-Iwan B, Mannuelian T, Skerka C, Lenk D, Bender BU, Cybulla M, Riegler P, Konigsrainer A, Neyer U, Bock A, Widmer U, Male DA, Franke G, Zipfel PF: Haemolytic uraemic syndrome and mutations of the factor H gene: a registry-based study of German speaking countries. *J Med Genet* 40:676-681, 2003

87. Dragon-Durey MA, Fremeaux-Bacchi V, Loirat C, Blouin J, Niaudet P, Deschenes G, Coppo P, Herman Fridman W, Weiss L: Heterozygous and homozygous factor h deficiencies associated with hemolytic uremic syndrome or membranoproliferative glomerulonephritis: report and genetic analysis of 16 cases. *J Am Soc Nephrol* 15:787-795, 2004

88. Perez-Caballero D, Gonzalez-Rubio C, Gallardo ME, Vera M, Lopez-Trascasa M, Rodriguez de Cordoba S, Sanchez-Corral P: Clustering of missense mutations in the C-terminal region of factor H in atypical hemolytic uremic syndrome. *Am J Hum Genet* 68:478-484, 2001

89. Buddles MR, Donne RL, Richards A, Goodship J, Goodship TH: Complement factor H gene mutation associated with autosomal recessive atypical hemolytic uremic syndrome. *Am J Hum Genet* 66:1721-1722, 2000

90. Sanchez-Corral P, Perez-Caballero D, Huarte O, Simckes AM, Goicoechea E, Lopez-Trascasa M, de Cordoba SR: Structural and functional characterization of factor H mutations associated with atypical hemolytic uremic syndrome. *Am J Hum Genet* 71:1285-1295, 2002

91. Jozsi M, Manuelian T, Heinen S, Oppermann M, Zipfel PF: Attachment of the soluble complement regulator factor H to cell and tissue surfaces: relevance for pathology. *Histol Histopathol* 19:251-258, 2004

92. Remuzzi G: Is ADAMTS-13 deficiency specific for thrombotic thrombocytopenic purpura? No. *J Thromb Haemost* 1:632-634, 2003

93. Tsai HM: Is severe deficiency of ADAMTS-13 specific for thrombotic thrombocytopenic purpura? Yes. *J Thromb Haemost* 1:625-631, 2003

94. Noris M, Remuzzi G: Hemolytic uremic syndrome. *J Am Soc Nephrol* 16:1035-1050, 2005

95. Noris M, Brioschi S, Caprioli J, Todeschini M, Bresin E, Porrati F, Gamba S, Remuzzi G: Familial haemolytic uraemic syndrome and an MCP mutation. *Lancet* 362:1542-1547, 2003

96. Johnstone RW, Loveland BE, McKenzie IF: Identification and quantification of complement regulator CD46 on normal human tissues. *Immunology* 79:341-347, 1993

97. Barilla-LaBarca ML, Liszewski MK, Lambris JD, Hourcade D, Atkinson JP: Role of membrane cofactor protein (CD46) in regulation of C4b and C3b deposited on cells. *J Immunol* 168:6298-6304, 2002

98. Richards A, Kemp EJ, Liszewski MK, Goodship JA, Lampe AK, Decorte R, Muslumanoglu MH, Kavukcu S,

Filler G, Pirson Y, Wen LS, Atkinson JP, Goodship TH: Mutations in human complement regulator, membrane cofactor protein (CD46), predispose to development of familial hemolytic uremic syndrome. *Proc Natl Acad Sci U S A* 100:12966-12971, 2003

99. Gerber A, Kirchhoff-Moradpour AH, Obieglo S, Brandis M, Kirschfink M, Zipfel PF, Goodship JA, Zimmerhackl LB: Successful (?) therapy of hemolytic-uremic syndrome with factor H abnormality. *Pediatr Nephrol* 18:952-955, 2003

100. Filler G, Radhakrishnan S, Strain L, Hill A, Knoll G, Goodship TH: Challenges in the management of infantile factor H associated hemolytic uremic syndrome. *Pediatr Nephrol* 19:908-911, 2004

101. Stratton JD, Warwicker P: Successful treatment of factor H-related haemolytic uraemic syndrome. *Nephrol Dial Transplant* 17:684-685, 2002

102. Artz MA, Steenbergen EJ, Hoitsma AJ, Monnens LA, Wetzels JF: Renal transplantation in patients with hemolytic uremic syndrome: high rate of recurrence and increased incidence of acute rejections. *Transplantation* 76:821-826, 2003

103. Miller RB, Burke BA, Schmidt WJ, Gillingham KJ, Matas AJ, Mauer M, Kashtan CE: Recurrence of haemolytic-uraemic syndrome in renal transplants: a single-centre report. *Nephrol Dial Transplant* 12:1425-1430, 1997

104. Fremeaux-Bacchi V, Dragon-Durey MA, Blouin J, Vigneau C, Kuypers D, Boudailliez B, Loirat C, Rondeau E, Fridman WH: Complement factor I: a susceptibility gene for atypical haemolytic uraemic syndrome. *J Med Genet* 41:e84, 2004

105. Kavanagh D, Kemp EJ, Mayland E, Winney RJ, Duffield JS, Warwick G, Richards A, Ward R, Goodship JA, Goodship TH: Mutations in Complement Factor I Predispose to Development of Atypical Hemolytic Uremic Syndrome. *J Am Soc Nephrol*, 2005

106. Kirschfink M: Targeting complement in therapy. *Immunol Rev* 180:177-189, 2001

CHAPTER 9

Summary

Hemolytic uremic syndrome (HUS) is a rare disease of microangiopathic hemolytic anemia, low platelet count and renal impairment due to platelet thrombi occluding the renal circulation. In children, the disease is most commonly triggered by certain strains of *E. coli* that produce powerful exotoxins, the Shiga-like toxins (Stx1 and Stx2) and manifests with diarrhea, often bloody. Non-Shiga-toxin-associated HUS (non-Stx-HUS) comprises a heterogeneous group of patients in whom an infection by Stx-producing bacteria could be excluded as a cause of the disease and can be sporadic or familial. Collectively, non-Stx-HUS have a poor outcome, with death or permanent renal dysfunction being the final outcome in the large majority of cases. Since early seventies an underlying genetically determined condition predisposing to these forms of HUS has been hypothesized. Finding of depressed complement C3 levels and increased concentrations of C3 breakdown products in patient blood suggested an inherited defect causing hyperactivation of the complement cascade.

In this thesis a number of studies were designed and performed with the aims to provide more information as for the incidence and pathogenetic role of complement disregulation in non-Stx-HUS and to investigate the genetic basis of such abnormalities. Sporadic observations in literature provided evidence that activation of complement occurs also in patients with thrombotic thrombocytopenic purpura (TTP). The latter syndrome has in common with HUS microangiopathic hemolytic anemia, consumption thrombocytopenia and microvascular thrombosis, but differs because it manifests mainly with central nervous system symptom, whereas predominant renal involvement characterizes HUS. Examination of very large series of patients with HUS and TTP has pointed out that a neat clinical

distinction between the two syndromes is difficult. Thus, search for complement activation markers and of genetic abnormalities of complement regulatory genes was also undertaken in patients with diagnosis of TTP.

In **chapter 2**, in a case-control study by multivariate analysis, we correlated putative predisposing conditions, including low C3 serum levels, with history of disease in 15 cases reporting one or more episodes of familial HUS and TTP, in 25 age- and gender-matched healthy controls and in 63 case-relatives and 56 control-relatives, respectively. The relationship between history of disease, low C3, and factor H (CFH) abnormalities was investigated in all affected families and in 17 controls. Seventy-three percent of cases compared with 16% of controls ($P < 0.001$), and 24% of case-relatives compared with 5% of control-relatives ($P = 0.005$) had decreased C3 serum levels. At multivariate analysis, C3 serum level was the only parameter associated with the disease within affected families and in the overall study population. Thus, subjects with decreased C3 serum levels had a relative risk of HUS or TTP of 16.56 (95% confidence interval [CI], 1.66 to 162.39) within families and of 27.77 (95% CI, 2.44 to 314.19) in the overall population, compared to subjects with normal serum levels. CFH abnormalities were found in four of the cases, compared with three of the healthy family members ($P = 0.02$) and none of the controls ($P = 0.04$) and, within families, CFH abnormalities were correlated with C3 reduction ($P < 0.05$). These results indicate that reduced C3 clusters in familial HUS and TTP is likely related to a genetically determined deficiency in CFH and may predispose to the disease.

Studies of **chapters 3 and 4** were aimed at clarifying whether *CFH* mutations were

involved in genetic predisposition to non-Stx-HUS, by performing linkage and mutation studies in patients referred to the International Registry for Recurrent and Familial HUS/TTP. In **chapter 4**, five mutations in the *CFH* gene were identified. Three mutations, identified in two families and in a sporadic case, are heterozygous point mutations causing amino acid exchange within the most C-terminal short consensus repeat 20 (SCR20) of CFH, resulting in single amino acid substitutions. The other two mutations introduce premature stop codons that interrupt the translation of CFH. A heterozygous nonsense mutation was identified in SCR8 in one family, and a homozygous 24-bp deletion within SCR20 was identified in a Bedouin family with a recessive mode of inheritance. Reverse transcription-PCR analysis of cDNA from peripheral blood leukocytes from the Bedouin family showed that the deletion lowered CFH mRNA levels. Although heterozygous mutations were associated with normal CFH levels and incomplete penetrance of the disease, the homozygous mutation in the Bedouin family resulted in severe reduction of CFH levels accompanied by very early disease onset.

In **chapter 4**, we analyzed the complete *CFH* gene in 101 patients with non-Stx-HUS, in 32 with TTP and in 106 controls to evaluate the frequency of *CFH* mutations, the clinical outcome in mutation and non-mutation carriers and the role of *CFH* polymorphisms in the predisposition to HUS. We found 17 *CFH* mutations (16 heterozygous, one homozygous) in 33 HUS patients. Thirteen mutations were located in exons XXII and XXIII. The disease manifested earlier and the mortality rate was higher in mutation carriers than in non-carriers. Kidney transplants invariably failed for disease recurrences in patients with *CFH* mutations,

while in non-mutated patients half of the grafts were functioning after 1 year. Three *CFH* polymorphic variants were strongly associated with non-Stx-HUS: -257T (promoter region), 2089G (exon XIV, silent) and 2881T (963Asp, SCR16). The association was stronger in patients without *CFH* mutations. Two or three disease-associated variants led to a higher risk of HUS than a single one. Analysis of available relatives of mutated patients revealed a penetrance of 50%. In 5/9 families the proband inherited the mutation from one parent and two disease-associated variants from the other, while unaffected carriers inherited the protective variants. In conclusion *CFH* mutations are frequent in patients with non-Stx-HUS (24%). Common polymorphisms of *CFH* may contribute to non-Stx-HUS manifestation in subjects with and without *CFH* mutations.

These data provide compelling molecular evidence that genetically determined deficiencies in CFH are involved in both autosomal-dominant and autosomal-recessive HUS and identify SCR20 as a hot spot for mutations in the disease.

Of interest, in studies of **chapter 4**, none of the 32 patients with a diagnosis of TTP and no renal impairment carried *CFH* mutations, which indicates that CFH abnormalities are specifically associated with renal manifestations of the thrombotic microangiopathy.

Evidence is now available that most cases of TTP are triggered by deficiency of ADAMTS13, a plasma metalloprotease that cleaves von Willebrand factor multimers. ADAMTS13 is usually normal in HUS, however there are rare unequivocal cases of HUS characterized by low or undetectable ADAMTS13 activity. In **chapter 5** we investigated the genetic basis of phenotype heterogeneity in patients with ADAMTS13 deficiency in two

sisters within one family. The patients had ADAMTS13 deficiency as a result of two heterozygous mutations (causing V88M and G1239V changes). In addition, a heterozygous mutation (causing an S890I change) in *CFH* was found in the patient who developed chronic renal failure but not in her sister, who presented with exclusive neurologic symptoms. These data indicate that in the above patient CFH haploinsufficency had a role in determining the renal complication and the HUS phenotype that superimposed on the systemic disease caused by ADAMTS13 deficiency.

Two third of patients with non-Stx-HUS have no *CFH* mutations, despite decreased serum concentrations of C3. The aim of **chapter 6**, therefore, was to assess whether genetic abnormalities in other complement regulatory proteins are involved. We screened genes that encode the complement regulatory proteins- i.e. factor H related-5, complement receptor 1, and membrane cofactor protein (MCP)-by PCR-single-strand conformation polymorphism (PCR-SSCP) and by direct sequencing, in 25 consecutive patients with non-Stx-HUS, an abnormal complement profile, and no *CFH* mutation, from our International Registry of Recurrent and Familial HUS/TTP. We identified a heterozygous mutation in MCP, a surface-bound complement regulator, in two patients with a familial history of HUS. The mutation causes a change in three aminoacids at position 233-35 and insertion of a premature stop-codon, which results in loss of the transmembrane domain of the protein and severely reduced cell-surface expression of MCP. Findings of an MCP mutation in two related patients suggest that impaired regulation of complement activation might be a factor in the pathogenesis of genetic forms of HUS. MCP could be a second

putative candidate gene for non-Stx-HUS. The protein is highly expressed in the kidney and plays a major part in regulation of glomerular C3 activation. We propose, therefore, that reduced expression of MCP in response to complement-activating stimuli could prevent restriction of complement deposition on glomerular endothelial cells, leading to microvascular cell damage and tissue injury.

Finally the review **chapter 7**, was aimed at summarize the more recent advances from our and form other groups on the pathogenesis, the genetics and treatment of different forms of HUS.

Curriculum Vitae

Marina Noris was born in Alzano Lombardo (BG), Italy in 1961. She achieved her University degree in Pharmaceutical Chemistry at the University of Rome "La Sapienza" in 1986. From 1986 to 1987 she was a post doctoral fellow of the Institute of Pharmaceutical Chemistry at the University of Rome. In September 1987 she began her career at the Mario Negri Institute in Bergamo participating to the unique group of basic scientists and clinicians working with professor Giuseppe Remuzzi in the field of renal disease progression and transplant immunology. From 1994 to 1996 she was head of the Unit of Endothelial Cell Pathophysiology, from 1996 to 1999 she was head of the Laboratory of Cellular and Molecular Biology of the Immune Response and Autoimmunity at Mario Negri Institute in Bergamo. From January 2000 she become head of the Laboratory of Immunology and Genetics of Transplantation and Rare Diseases at the Clinical Research Center for Rare Diseases in Ranica of the Mario Negri Institute). Dr. Noris has authored or co-authored over 90 articles for publication in scientifc journals, books, etc. expecially in the field of pathophysiology and genetics of hemolytic uremic syndrome and thrombotic thrombocytopenic purpura and in the field of experimental transplant immunology. She has also undertaken several studies on nitric oxide and oxygen radical as uremic toxins and in particular on the role of nitric oxide in uremic bleeding and dialysis complications.

Acknowledgments

I am very indebted and grateful to my mentor Prof. Giuseppe Remuzzi. He encouraged me since the beginning to pursue the research work and continuously favored my intellectual growth. He was always available to discuss the results as well strategies to achieve a real integration between basic and clinical science.

I gratefully acknowledge Prof. Karel Leunissen for the opportunity he gave me to discuss my PhD thesis in the prestigious Universiteit Maastricht.

I am also very grateful to students, technicians and graduate fellows in my lab who contributed with their work and ideas to the results presented in this thesis.

Finally I wish to acknowledge my husband Daniele who has always supported my research work and daily encourages me to keep on and to overcome obstacles.